Comparison of the B72.3 murine and EU heavy chain sequences reveals that the mouse and human residues are identical at positions 23, 24, 71 and 78.
Thus the mutated CDR-grafted B72.3 heavy chain corresponds to a preferred embodiment of the present invention.

EXAMPLE 4

CDR-GRAFTING OF A MURINE ANTI-ICAM-1 MONOCLONAL ANTIBODY

A murine antibody, R6-5-D6 (EP 0314863) having specificity for Intercellular Adhesion Molecule 1 (ICAM-1) was CDR-grafted substantially as described above in previous examples. This work is described in greater detail in co-pending application, British Patent Application No. 9009549.8, the disclosure of which is incorporated herein by reference.

The human EU framework was used as the acceptor framework for both heavy and light chains. The CDR-grafted antibody currently of choice is provided by co-expression of grafted light chain gL221A and grafted heavy chain gH341D which has a binding affinity for ICAM 1 of about 75% of that of the corresponding mouse-human chimeric antibody.

LIGHT CHAIN

gL221A has murine CDRs at positions 24-34 (CDR1), 50-56 (CDR2) and 89-97 (CDR3). In addition several framework residues are also the murine amino acid. These residues were chosen after consideration of the possible contribution of these residues to domain packing and stability of the conformation of the antigen binding region. The residues which have been retained as mouse are at positions 2, 3, 48 (?), 60, 84, 85 and 87. Comparison of the murine anti-ICAM 1 and human EU light chain amino acid sequences reveals that the murine and human residues are identical at positions 46, 58 and 71.

HEAVY CHAIN

gH341D has murine CDRs at positions 26-35 (CDR1), 50-56 (CDR2) and94-100B (CDR3). In addition murine residues were used in gH341D at positions 24, 48, 69, 71, 73, 80, 88 and 91. Comparison of the murine anti-ICAM 1 and human EU heavy chain amino acid sequences are identical at positions 23, 49 and 78.

EXAMPLE 5

CDR-Grafting of murine anti-TNFα antibodies

A number of murine anti-TNFα monoclonal antibodies were CDR-grafted substantially as described above in previous examples. These antibodies include the murine monoclonal antibodies designated 61 E71, hTNF1, hTNF3 and 101.4 A brief summary of the CDR-grafting of each of these antibodies is given below.

61E71

A similar analysis as described above (Example 1, Section 12.1.) was done for 61E71 and for the heavy chain 10 residues were identified at 23, 24, 48, 49, 68, 69, 71, 73, 75 and 88 as residues to potentially retain as murine. The human frameworks chosen for CDR-grafting of this antibody, and the hTNF3 and 101.4 antibodies were RE1 for the light chain and KOL for the heavy chain.
Three genes were built, the first of which contained 23, 24, 48, 49, 71 and 73 [gH341(6)] as murine residues. The second gene also had 75 and 88 as murine residues [gH341(8)] while the third gene additionally had 68, 69, 75 and 88 as murine residues [gH341(10)]. Each was co-expressed with gL221, the minimum grafted light chain (CDRs only). The gL221/gH341(6) and gL221/gH341(8) antibodies both bound as well to TNF as murine 61E71. The gL221/gH341(10) antibody did not express and this combination was not taken further.
Subsequently the gL221/gH341(6) antibody was assessed in an L929 cell competition assay in which the antibody competes against the TNF receptor on L929 cells for binding to TNF in solution. In this assay the gL221/gH341(6) antibody was approximately 10% as active as murine 61E71.

hTNF1

hTNF1 is a monoclonal antibody which recognises an epitope on human TNF- . The EU human framework was used for CDR-grafting of both the heavy and light variable domains.

Heavy Chain

In the CDR-grafted heavy chain (ghTNF1) mouse CDRs were used at positions 26-35 (CDR1), 50-65 (CDR2) and 95-102 (CDR3). Mouse residues were also used in the frameworks at positions 48, 67, 69, 71, 73, 76, 89, 91, 94 and 108. Comparison of the TNF1 mouse and EU human heavy chain residues reveals that these are identical at positions 23, 24, 29 and 78.

Light Chain

In the CDR-grafted light chain (gLhTNF1) mouse CDRs wre used at positions 24-34 (CDR1), 50-56 (CDR2) and 89-97 (CDR3). In addition mouse residues were used in the frameworks at positions 3, 42, 48, 49, 83, 106 and 108. Comparison of the hTNF1 mouse and EU human light chain residues reveals that these are identical at positions 46, 58 and 71.

The grafted hTNF1 heavy chain was co-expressed with the chimeric light chain and the binding ability of the product compared with that of the chimeric light chain/chimeric heavy chain product in a TNF binding assay. The grafted heavy chain product appeared to have binding ability for TNF slightly better than the fully chimeric product.

Similarly, a grafted heavy chain/grafted light chain product was co-expressed and compared with the fully chimeric product and found to have closely similar binding properties to the latter product.

hTNF3

hTNF3 recognises an epitope on human TNF-α. The sequence of hTNF3 shows only 21 differences compared to 61E71 in the light and heavy chain variable regions, 10 in the light chain (2 in the CDRs at positions 50, 96 and 8 in the framework at 1, 19, 40, 45, 46, 76, 103 and 106) and 11 in the heavy chain (3 in the CDR regions at positions 52, 60 and 95 and 8 in the framework at 1, 10, 38, 40, 67, 73, 87 and 105). The light and heavy chains of the 61E71 and hTNF3 chimeric antibodies can be exchanged without loss of activity in the direct binding assay. However 61E71 is an order of magnitude less able to compete with the TNF receptor on L929 cells for TNF-a compared to hTNF3. Based on the 61E71 CDR grafting data gL221 and gH341(+23, 24, 48, 49 71 and 73 as mouse) genes have been built for hTNF3 and tested and the resultant grafted antibody binds well to TNF-a, but competes very poorly in the L929 assay. It is possible that in this case also the framework residues identified for OKT3 programme may improve the competitive binding ability of this antibody.

101.4

101.4 is a further murine monoclonal antibody able to recognise human TNF-a. The heavy chain of this antibody shows good homology to KOL and so the CDR-grafting has been based on RE1 for the light chain and KOL for the heavy chain. Several grafted heavy chain genes have been constructed with conservative choices for the CDR's (gH341) and which have one or a small number of non-CDR residues at positions 73, 78 or 77-79 inclusive, as the mouse amino acids. These have been co-expressed with cL or gL221. In all cases binding to TNF equivalent to the chimeric antibody is seen and when co-expressed with cL the resultant antibodies are able to compete well in the L929 assay. However, with gL221 the resultant antibodies

are at least an order of magnitude less able to compete for TNF against the TNF receptor on L929 cells.

Mouse residues at other positions in the heavy chain, for example, at 23 and 24 together or at 76 have been demonstrated to provide no improvement to the competitive ability of the grafted antibody in the L929 assay.

A number of other antibodies including antibodies having specificity for interleukins e.g. IL1 and cancer markers such as carcinoembryonic antigen (CEA) e.g. the monoclonal antibody A5B7 (ref. 21), have been successfully CDR-grafted according to the present invention.
It will be appreciated that the foregoing examples are given by way of illustration only and are not intended to limit the scope of the claimed invention. Changes and modifications may be made to the methods described whilst still falling within the spirit and scope of the invention.

References

1. Kohler & Milstein, Nature, 265, 295-497, 1975.

2. Chatenoud et al, (1986), J. Immunol. 137, 830-838.

3. Jeffers et al, (1986), Transplantation, 41, 572-578.

4. Begent et al, Br. J. Cancer 62: 487 (1990).

5. Verhoeyen et al, Science, 239, 1534-1536, 1988.

6. Riechmann et al, Nature, 332, 323-324, 1988.

7. Kabat, E.A., Wu, T.T., Reid-Miller, M., Perry, H.M., Gottesman, K.S., 1987, in Sequences of Proteins of Immunological Interest, US Department of Health and Human Services, NIH, USA.

8. Wu, T.T., and Kabat, E.A., 1970, J. Exp. Med. 132 211-250.

9. Queen et al, (1989), Proc. Natl. Acad. Sci. USA, 86, 10029-10033 and WO 90/07861

10. Maniatis et al, Molecular Cloning, Cold Spring Harbor, New York, 1989.

11. Primrose and Old, Principles of Gene Manipulation, Blackwell, Oxford, 1980.

12. Sanger, F., Nicklen, S., Coulson, A.R., 1977, Proc. Natl. Acad. Sci. USA, 74 5463

13. Kramer, W., Drutsa, V., Jansen, H.-W., Kramer, B., Plugfelder, M., Fritz, H.-J., 1984, Nucl. Acids Res. 12, 9441

14. Whittle, N., Adair, J., Lloyd, J.C., Jenkins, E., Devine, J., Schlom, J., Raubitshek, A., Colcher, D., Bodmer, M., 1987, Protein Engineering 1, 499.

15. Sikder, S.S., Akolkar, P.N., Kaledas, P.M., Morrison, S.L., Kabat, E.A., 1985, J. Immunol. 135, 4215.

16. Wallick, S.C., Kabat, E.A., Morrison, S.L., 1988, J. Exp. Med. 168, 1099

17. Bebbington, C.R., Published International Patent Application WO 89/01036.

18. Granthan and Perrin 1986, Immunology Today 7, 160.

19. Kozak, M., 1987, J. Mol. Biol. 196, 947.

20. Jones, T.P., Dear, P.H., Foote, J., Neuberger, M.S., Winter, G., 1986, Nature, 321, 522

21. Harwood et al, Br. J. Cancer, 54, 75-82 (1986).

CLAIMS

1. A CDR-grafted antibody heavy chain having a variable region domain comprising acceptor framework and donor antigen binding regions wherein the framework comprises donor residues at at least one of positions 6, 23 and/or 24, 48 and/or 49, 71 and/or 73, 75 and/or 76 and/or 78 and 88 and/or 91.

2. A CDR-grafted heavy chain according to Claim 1 comprising donor residues at positions 23, 24, 49, 71, 73 and 78, or at positions 23, 24 and 49.

3. A CDR-grafted heavy chain according to Claim 2 comprising donor residues at positions 2, 4, 6, 25, 36, 37, 39, 47, 48, 93, 94, 103, 104, 106 and 107.

4. A CDR-grafted heavy chain according to Claim 2 or 3, comprising donor residues at one, some or all of positions:
1 and 3,
69 (if 48 is different between donor and acceptor),
38 and 46 (if 48 is the donor residue),
67,
82 and 18 (if 67 is the donor residue),
91, and
any one or more of 9, 11, 41, 87, 108, 110 and 112.

5. A CDR-grafted heavy chain according to any of the preceding comprising donor CDRs at positions 26-35, 50-65 and 95-100.

6. A CDR-grafted antibody light chain having a variable region domain comprising acceptor framework and donor antigen binding regions wherein the framework comprises donor residues at at least one of positions 1 and/or 3 and 46 and/or 47.

7. A CDR-grafted light chain according to Claim 6 comprising donor residues at positions 46 and 47.

8. A CDR-grafted antibody light chain having a variable region domain comprising acceptor framework and donor antigen binding regions wherein the framework comprises donor residues at at least one of positions 46, 48, 58 and 71.

9. A CDR-grafted light chain according to Claim 8 comprising donor residues at positions 46, 48, 58 and 71.

10. A CDR-grafted light chain according to Claim 8 or 9, comprising donor residues at positions 2, 4, 6, 35, 36, 38, 44, 47, 49, 62, 64-69, 85, 87, 98, 99, 101 and 102.

11. A CDR-grafted light chain according to Claim 9 or 10, comprising donor residues at one, some or all of positions:
1 and 3,
63,
60 (if 60 and 54 are able to form a potential saltbridge),
70 (if 70 and 24 are able to form a potential saltbridge),
73 and 21 (if 47 is different between donor and acceptor),
37 and 45 (if 47 if different between donor and acceptor), and
any one or more of 10, 12, 40, 83, 103 and 105.

12. A CDR-grafted light chain according to any one of Claims 6-11, comprising donor CDRs at positions 24-34, 50-56 and 89-97.

13. A CDR-grafted antibody molecule comprising at least one CDR-grafted heavy chain according to any one of Claims 1-5 and at least one CDR-grafted light chain according to any one of Claims 6-12.

14. A CDR-grafted antibody molecule according to Claim 13, which is a site-specific antibody molecule.

15. A CDR-grafted antibody molecule according to Claim 13 which has specificity for an interleukin, hormone or other biologically active compound or a receptor therefor.

16. A CDR-grafted antibody heavy or light chain or molecule according to any one of the preceding claims comprising human acceptor residues and non-human donor residues.

17. A DNA sequence which codes for a CDR-grafted heavy chain according to Claim 1 or a CDR-grafted light chain according to Claim 6 or Claim 8.

18. A cloning or expression vector containing a DNA sequence according to Claim 17.

19. A host cell transformed with a DNA sequence according to Claim 17.

20. A process for the production of a CDR-grafted antibody sequence according to Claim 17 in a transformed host cell.

21. A process for producing a CDR-grafted antibody product comprising:

(a) producing in an expression vector an operon having a DNA sequence which encodes an antibody heavy chain according to Claim 1;

and/or

(b) producing in an expression vector an operon having a DNA sequence which encodes a complementary antibody light chain according to Claim 6 or Claim 8;

(c) transfecting a host cell with the or each vector; and

(d) culturing the transfected cell line to produce the CDR-grafted antibody product.

22. A therapeutic or diagnostic composition comprising a CDR-grafted antibody heavy chain according to Claim 1, or a CDR-grafted light chain according to Claim 6 or Claim 8, or a CDR-grafted antibody molecule according to Claim 13 in combination with a pharmaceutically acceptable carrier, diluent or excipient.

23. A method of therapy or diagnosis comprising administering an effective amount of a CDR-grafted heavy chain according to Claim 1, or a CDR-grafted light chain according to Claim 6 or Claim 8, or a CDR-grafted antibody molecule according to Claim 13 to a human or animal subject.

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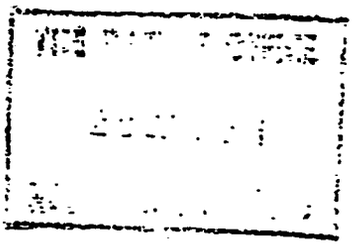

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23rd January, 1991.

REQUEST FOR RECTIFICATION UNDER PCT RULE 91.1(f)

Dear Sirs,

Re: International Patent Application No. PCT/GB90/02017
Celltech Limited et al.

I refer to your Invitation issued on 14th January 1991. The required Authorisations and Formal Drawings will be filed in due course.

In checking the application, it has become apparent that there are three mistakes in the Request Form.

Firstly,....

Secondly,....

Thirdly, for reasons which are not apparent, an old version of the Request Form (PCT/RO/101 of July 1987) was used instead of the most up-to-date version. As a result of this, some PCT states were not designated although it was the Applicant's intention that all possible states sh uld have been designated. As evidence of this, I attach a copy of the information sheet which was given to me by hand by the Applicant's Patent Manager on the date the application

was filed. It can be seen that this clearly indicates that all territories should have been designated.

I also enclose evidence that the out-of-date Request Form was used inadvertently. At the same time as the present application was filed, I also filed two other PCT applications, Nos. PCT/GB90/02015 and PCT/GB90/02018. I enclose copies of the Request Forms for these cases which, as you can see, are the most up-to-date versions of the forms.

I therefore request that the Request Form be amended by adding thereto the designations of Canada and Spain as national applications and Greece, Spain and Denmark as designated states within the EPC designation. I note that it will not be necessary to pay any extra fees in respect of these inadvertently omitted designations.

In order to effect all these corrections, I enclose a retyped, up-to-date (at the date of filing) Request Form and request that this be substituted for the present, out-of-date Request Form.

Yours truly,

MERCER, Christopher Paul
Authorised Representative.

```
   1  GAATTCCCAA AGACAAAatg gattttcaag tgcagatttt cagcttcctg
  51  ctaatcagtg cctcagtcat aatatccaga ggacaaattg ttctcaccca
 101  gtctccagca atcatgtctg catctccagg ggagaaggtc accatgacct
 151  gcagtgccag ctcaagtgta agttacatga actggtacca gcagaagtca
 201  ggcacctccc ccaaaagatg gatttatgac acatccaaac tggcttctgg
 251  agtccctgct cacttcaggg gcagtgggtc tgggacctct tactctctca
 301  caatcagcgg catggaggct gaagatgctg ccacttatta ctgccagcag
 351  tggagtagta acccattcac gttcggctcg gggacaaagt tggaaataaa
 401  ccgggctgat actgcaccaa ctgtatccat cttcccacca tccagtgagc
 451  agttaacatc tggaggtgcc tcagtcgtgt gcttcttgaa caacttctac
 501  cccaaagaca tcaatgtcaa gtggaagatt gatggcagtg aacgacaaaa
 551  tggcgtcctg aacagttgga ctgatcagga cagcaaagac agcacctaca
 601  gcatgagcag caccctcacg ttgaccaagg acgagtatga acgacataac
 651  agctatacct gtgaggccac tcacaagaca tcaacttcac ccattgtcaa
 701  gagcttcaac aggaatgagt gtTAGAGACA AAGGTCCTGA GACGCCACCA
 751  CCAGCTCCCA GCTCCATCCT ATCTTCCCTT CTAAGGTCTT GGAGGCTTCC
 801  CCACAAGCGC tTACCACTGT TGCGGTGCTC tAAACCTCCT CCCACCTCCT
 851  TCTCCTCCTC CTCCCTTTCC TTGGCTTTTA TCATGCTAAT ATTTGCAGAA
 901  AATATTCAAT AAAGTGAGTC TTTGCCTTGA AAAAAAAAAA AAA
```

Fig. 1(a)

```
   1  MDFOVOIFSF LLISASVIIS RGQIVLTQSP AIMSASPGEK VTMTCSASSS
  51  VSYMNWYQQK SGTSPKRWIY DTSKLASGVP AHFRGSGSGT SYSLTISGME
 101  AEDAATYYCQ QWSSNPFTFG SGTKLEINRA DTAPTVSIFP PSSEQLTSGG
 151  ASVVCFLNNF YPKDINVKWK IDGSERQNGV LNSWTDQDSK DSTYSMSSTL
 201  TLTKDEYERH NSYTCEATHK TSTSPIVKSF NRNEC*
```

Fig. 1(b)

```
   1 GAATTCCCCT CTCCACAGAC ACTGAAAACT CTGACTCAAC ATGGAAAGGC
  51 ACTGGATCTT TCTACTCCTG TTGTCAGTAA CTGCAGGTGT CCACTCCCAG
 101 GTCCAGCTGC AGCAGTCTGG GGCTGAACTG GCAAGACCTG GGGCCTCAGT
 151 GAAGATGTCC TGCAAGGCTT CTGGCTACAC CTTTACTAGG TACACGATGC
 201 ACTGGGTAAA ACAGAGGCCT GGACAGGGTC TGGAATGGAT TGGATACATT
 251 AATCCTAGCC GTGGTTATAC TAATTACAAT CAGAAGTTCA AGGACAAGGC
 301 CACATTGACT ACAGACAAAT CCTCCAGCAC AGCCTACATG CAACTGAGCA
 351 GCCTGACATC TGAGGACTCT GCAGTCTATT ACTGTGCAAG ATATTATGAT
 401 GATCATTACT GCCTTGACTA CTGGGGCCAA GGCACCACTC TCACAGTCTC
 451 CTCAGCCAAA ACAACAGCCC CATCGGTCTA TCCACTGGCC CCTGTGTGTG
 501 GAGATACAAC TGGCTCCTCG GTGACTCTAG GATGCCTGGT CAAGGGTTAT
 551 TTCCCTGAGC CAGTGACCTT GACCTGGAAC TCTGGATCCC TGTCCAGTGG
 601 TGTGCACACC TTCCCAGCTG TCCTGCAGTC TGACCTCTAC ACCCTCAGCA
 651 GCTCAGTGAC TGTAACCTCG AGCACCTGGC CCAGCCAGTC CATCACCTGC
 701 AATGTGGCCC ACCCGGCAAG CAGCACCAAG GTGGACAAGA AAATTGAGCC
 751 CAGAGGGCCC ACAATCAAGC CCTGTCCTCC ATGCAAATGC CCAGCACCTA
 801 ACCTCTTGGG TGGACCATCC GTCTTCATCT TCCCTCCAAA GATCAAGGAT
 851 GTACTCATGA TCTCCCTGAG CCCCATAGTC ACATGTGTGG TGGTGGATGT
 901 GAGCGAGGAT GACCCAGATG TCCAGATCAG CTGGTTTGTG AACAACGTGG
 951 AAGTACACAC AGCTCAGACA CAAACCCATA GAGAGGATTA CAACAGTACT
1001 CTCCGGGTGG TCAGTGCCCT CCCCATCCAG CACCAGGACT GGATGAGTGG
1051 CAAGGAGTTC AAATGCAAGG TCAACAACAA AGACCTCCCA GCGCCCATCG
1101 AGAGAACCAT CTCAAAACCC AAAGGGTCAG TAAGAGCTCC ACAGGTATAT
1151 GTCTTGCCTC CACCAGAAGA AGAGATGACT AAGAAACAGG TCACTCTGAC
1201 CTGCATGGTC ACAGACTTCA TGCCTGAAGA CATTTACGTG GAGTGGACCA
1251 ACAACGGGAA AACAGAGCTA AACTACAAGA ACACTGAACC AGTCCTGGAC
1301 TCTGATGGTT CTTACTTCAT GTACAGCAAG CTGAGAGTGG AAAAGAAGAA
1351 CTGGGTGGAA AGAAATAGCT ACTCCTGTTC AGTGGTCCAC GAGGGTCTGC
1401 ACAATCACCA CACGACTAAG AGCTTCTCCC GGACTCCGGG TAAATGAGCT
1451 CAGCACCCAC AAAACTCTCA GGTCCAAAGA GACACCCACA CTCATCTCCA
1501 TGCTTCCCTT GTATAAATAA AGCACCCAGC AATGCCTGGG ACCATGTAAA
1551  AAAAAAAAAA AAAGGAATTC
```

Fig. 2(a)

OKT 3 HEAVY CHAIN PROTEIN SEQUENCE DEDUCED FROM DNA SEQUENCE

```
  1  MERHWIFLLL LSVTAGVHSQ VQLQQSGAEL ARPGASVKMS CKASGYTFTR
 51  YTMHWVKQRP GQGLEWIGYI NPSRGYTNYN QKFKDKATLT TDKSSSTAYM
101  QLSSLTSEDS AVYYCARYYD DHYCLDYWGQ GTTLTVSSAK TTAPSVYPLA
151  PVCGDTTGSS VTLGCLVKGY FPEPVTLTWN SGSLSSGVHT FPAVLQSDLY
201  TLSSSVTVTS STWPSQSITC NVAHPASSTK VDKKIEPRGP TIKPCPPCKC
251  PAPNLLGGPS VFIFPPKIKD VLMISLSPIV TCVVVDVSED DPDVQISWFV
301  NNVEVHTAQT QTHREDYNST LRVVSALPIQ HQDWMSGKEF KCKVNNKDLP
351  APIERTISKP KGSVRAPQVY VLPPPEEEMT KKQVTLTCMV TDFMPEDIYV
401  EWTNNGKTEL NYKNTEPVLD SDGSYFMYSK LRVEKKNWVE RNSYSCSVVH
451  EGLHNHHTTK SFSRTPGK*
```

Fig. 2(b)

```
                1                       23                      42
                NN      N                 N       N     N
RES TYPE        SBspSPESssBSbSsSssPSPSPsPSsse*s*p*Pi^ISsSe
Okt3vl          QIVLTQSPAIMSASPGEKVTMTCSASS.SVSYMNWYQQKSGT
REI             DIQMTQSPSSLSASVGDRVTITCQASQDIIKYLNWYQQTPGK
                ? ?
                  CDR1    (LOOP)          *******
                  CDR1    (KABAT)        ***********


                          56                              85
                N  NN
RES TYPE        *IsiPpIeesesssSBEsePsPSBSSEsPspsPsseesSPePb
Okt3vl          SPKRWIYDTSKLASGVPAHFRGSGSGTSYSLTISGMEAEDAAT
REI             APKLLIYEASNLQAGVPSRFSGSGSGTDYTFTISSLQPEDIAT
                ?  ??                          ?  ?
                      *******  CDR2 (LOOP/KABAT)


                             102   108
RES TYPE        PiPIPies**iPIIsPPSPSPSS
Okt3vl          YYCQQWSSNPFTFGSGTKLEINR
REIvl           YYCQQYQSLPYTFGQGTKLQITR
                             ?    ?
                    ******        CDR3 (LOOP)
                  *********       CRD3 (KABAT)
```

Fig. 3

SUBSTITUTE SHEET

```
          NN N                  23 26    32 35 N39  43
RES TYPE  SESPs^SBssS^sSSsSpSpSPsPSEbSBssBePiPIpiesss
Okt3h     QVQLQQSGAELARPGASVKMSCKASGYTFTRYTMHWVKQRPGQ
KOL       QVQLVESGGGVVQPGRSLRLSCSSSGFIFSSYAMYWVRQAPGK
               ?                ??
                                     ******    CDR1 (LOOP)
                                        ***** CDR1 (KABAT)

                  52a      60  65     N N  N    82abc     89
RES TYPE IIeIppp^sssssssss^ps^pSSsbSpseSsSseSp^pSpsSBssS^ePb
Okt3vh   GLEWIGYINPSRGYTNTNQKFKDKATLTTDKSSSTAYMQLSSLTSEDSAV
KOL      GLEWVAIIWDDGSDQHYADSVKGRFTISRDNSKNTLFLQMDSLRPEDTGV
            ??                   ? ?  ?  ?             ?
               ************         CDR2  (LOOP)
               ******************** CDR2  (KABAT)

          92 N                   107    113
RES TYPE  PiPIEissssiiisssbibi*EIPIP*spSBSS
Okt3vh    YYCARYYDDHY.......CLDYWGQGTTLTVSS
KOL       YFCARDGGHGFCSSASCFGPDYWGQGTPVTVSS
               ****************** CRD3 (KABAT/LOOP)
```

Fig. 4

OKT 3 HEAVY CHAIN CDR GRAFTS

1. gh341 and derivatives

	1 26 35 39 43	
Okt3vh	QVQLQQSGAELARPGASVKMSCKASGYTFTRYTMHWVKQRPGQ	
gH341	QVQLVESGGGVVQPGRSLRLSCSSSGYTFTRYTMHWVRQAPGK	JA178
gH341A	QVQLVQSGGGVVQPGRSLRLSCKASGYTFTRYTMHWVRQAPGK	JA185
gH341E	QVQLVQSGGGVVQPGRSLRLSCKASGYTFTRYTMHWVRQAPGK	JA198
gH341*	QVQLVQSGGGVVQPGRSLRLSCKASGYTFTRYTMHWVRQAPGK	JA207
gH341*	QVQLVQSGGGVVQPGRSLRLSCKASGYTFTRYTMHWVRQAPGK	JA209
gH341D	QVQLVQSGGGVVQPGRSLRLSCKASGYTFTRYTMHWVRQAPGK	JA197
gH341*	QVQLVQSGGGVVQPGRSLRLSCKASGYTFTRYTMHWVRQAPGK	JA199
gH341C	QVQLVQSGGGVVQPGRSLRLSCKASGYTFTRYTMHWVRQAPGK	JA184
gH341*	QVQLVQSGGGVVQPGRSLRLSCSASGYTFTRYTMHWVRQAPGK	JA203
gH341*	QVQLVESGGGVVQPGRSLRLSCSASGYTFTRYTMHWVRQAPGK	JA205
gH341B	QVQLVESGGGVVQPGRSLRLSCSSSGYTFTRYTMHWVRQAPGK	JA183
gH341*	QVQLVQSGGGVVQPGRSLRLSCSASGYTFTRYTMHWVRQAPGK	JA204
gH341*	QVQLVESGGGVVQPGRSLRLSCSASGYTFTRYTMHWVRQAPGK	JA206
gH341*	QVQLVQSGGGVVQPGRSLRLSCSASGYTFTRYTMHWVRQAPGK	JA208
KOL	QVQLVESGGGVVQPGRSLRLSCSSSGFIFSSYAMYWVRQAPGK	

Fig. 5(i)

	44 50 65 83	
Okt3vh	GLEWIGYINPSRGYTNYNQKFKDKATLTTDKSSSTAYMQLSSLT	
gH341	GLEWVAYINPSRGYTNYNQKFKDRFTISRDNSKNTLFLQMDSLR	JA178
gH341A	GLEWIGYINPSRGYTNYNQKVKDRFTISTDKSKSTAFLQMDSLR	JA185
gH341E	GLEWIGYINPSRGYTNYNQKVKDRFTISTDKSKSTAFLQMDSLR	JA198
gH341*	GLEWIGYINPSRGYTNYNQKVKDRFTISTDKSKNTAFLQMDSLR	JA207
gH341*	GLEWIGYINPSRGYTNYNQKVKDRFTISRDNSKNTAFLQMDSLR	JA209
gH341D	GLEWIGYINPSRGYTNYNQKVKDRFTISTDKSKNTLFLQMDSLR	JA197
gH341*	GLEWIGYINPSRGYTNYNQKVKDRFTISRDNSKNTLFLQMDSLR	JA199
gH341C	GLEWVAYINPSRGYTNYNQKFKDRFTISRDNSKNTLFLQMDSLR	JA184
gH341*	GLEWIGYINPSRGYTNYNQKVKDRFTISTDKSKSTAFLQMDSLR	JA207
gH341*	GLEWIGYINPSRGYTNYNQKVKDRFTISTDKSKSTAFLQMDSLR	JA205
gH341B	GLEWIGYINPSRGYTNYNQKVKDRFTISTDKSKSTAFLQMDSLR	JA183
gH341*	GLEWIGYINPSRGYTNYNQKVKDRFTISTDKSKSTAFLQMDSLR	JA204
gH341*	GLEWIGYINPSRGYTNYNQKVKDRFTISTDKSKSTAFLQMDSLR	JA206
gH341*	GLEWIGYINPSRGYTNYNQKVKDRFTISTDKSKNTAFLQMDSLR	JA208
KOL	GLEWVAIIWDDGSDQHYADSVKGRFTISRDNSKNTLFLQMDSLR	

Fig. 5(ii)

	84 95 102 113	
Okt3vh	SEDSAVYYCARYYDDHY.....CLDYWGQGTTLTVSS	
gH341	PEDTGVYFCARYYDDHY.....CLDYWGQGTTLTVSS	JA178
gH341A	PEDTAVYYCARYYDDHY.....CLDYWGQGTTLTVSS	JA185
gH341E	PEDTGVYFCARYYDDHY.....CLDYWGQGTTLTVSS	JA198
gH341*	PEDTGVYFCARYYDDHY.....CLDYWGQGTTLTVSS	JA207
gH341D	PEDTGVYFCARYYDDHY.....CLDYWGQGTTLTVSS	JA197
gH341*	PEDTGVYFCARYYDDHY.....CLDYWGQGTTLTVSS	JA209
gH341*	PEDTGVYFCARYYDDHY.....CLDYWGQGTTLTVSS	JA199
gH341C	PEDTGVYFCARYYDDHY.....CLDYWGQGTTLTVSS	JA184
gH341*	PEDTAVYYCARYYDDHY.....CLDYWGQGTTLTVSS	JA203
gH341*	PEDTAVYYCARYYDDHY.....CLDYWGQGTTLTVSS	JA205
gH341B	PEDTAVYYCARYYDDHY.....CLDYWGQGTTLTVSS	JA183
gH341*	PEDTGVYFCARYYDDHY.....CLDYWGQGTTLTVSS	JA204
gH341*	PEDTGVYFCARYYDDHY.....CLDYWGQGTTLTVSS	JA206
gH341*	PEDTGVYFCARYYDDHY.....CLDYWGQGTTLTVSS	JA208
KOL	PEDTGVYFCARDGGHGFCSSASCFGPDYWGQGTPVTVSS	

Fig. 5(iii)

OKT3 LIGHT CHAIN CDR GRAFTING

1. gL221 and derivatives

```
          1                        24        34       42
Okt3vl    QIVLTQSPAIMSASPGEKVTMTCSASS.SVSYMNWYQQKSGT
gL221     DIQMTQSPSSLSASVGDRVTITCSASS.SVSYMNWYQQTPGK
gL221A    QIVMTQSPSSLSASVGDRVTITCSASS.SVSYMNWYQQTPGK
gL221B    QIVMTQSPSSLSASVGDRVTITCSASS.SVSYMNWYQQTPGK
gL221C    DIQMTQSPSSLSASVGDRVTITCSASS.SVSYMNWYQQTPGK
REI       DIQMTQSPSSLSASVGDRVTITCQASQDIIKYLNWYQQTPGK

          43      50     56                          85
Okt3vl    SPKRWIYDTSKLASGVPAHFRGSGSGTSYSLTISGMEAEDAAT
gL221     APKLLIYDTSKLASGVPSRFSGSGSGTDYTFTISSLQPEDIAT
gL221A    APKRWIYDTSKLASGVPSRFSGSGSGTDYTFTISSLQPEDIAT
gL221B    APKRWIYDTSKLASGVPSRFSGSGSGTDYTFTISSLQPEDIAT
gL221C    APKRWIYDTSKLASGVPSRFSGSGSGTDYTFTISSLQPEDIAT
REI       APKLLIYEASNLQAGVPSRFSGSGSGTDYTFTISSLQPEDIAT

          86   91   96          108
Okt3vl    YYCQQWSSNPFTFGSGTKLEINR
gL221     YYCQQWSSNPFTFGQGTKLQITR
gL221A    YYCQQWSSNPFTFGQGTKLQITR
gL221B    YYCQQWSSNPFTFGQGTKLQITR
gL221C    YYCQQWSSNPFTFGQGTKLQITR
REI       YYCQQYQSLPYTFGQGTKLQITR
```

CDR'S ARE UNDERLINED

FRAMEWORK RESIDUES INCLUDED IN THE GENE ARE DOUBLE UNDERLINED

Fig. 6

Fig. 7

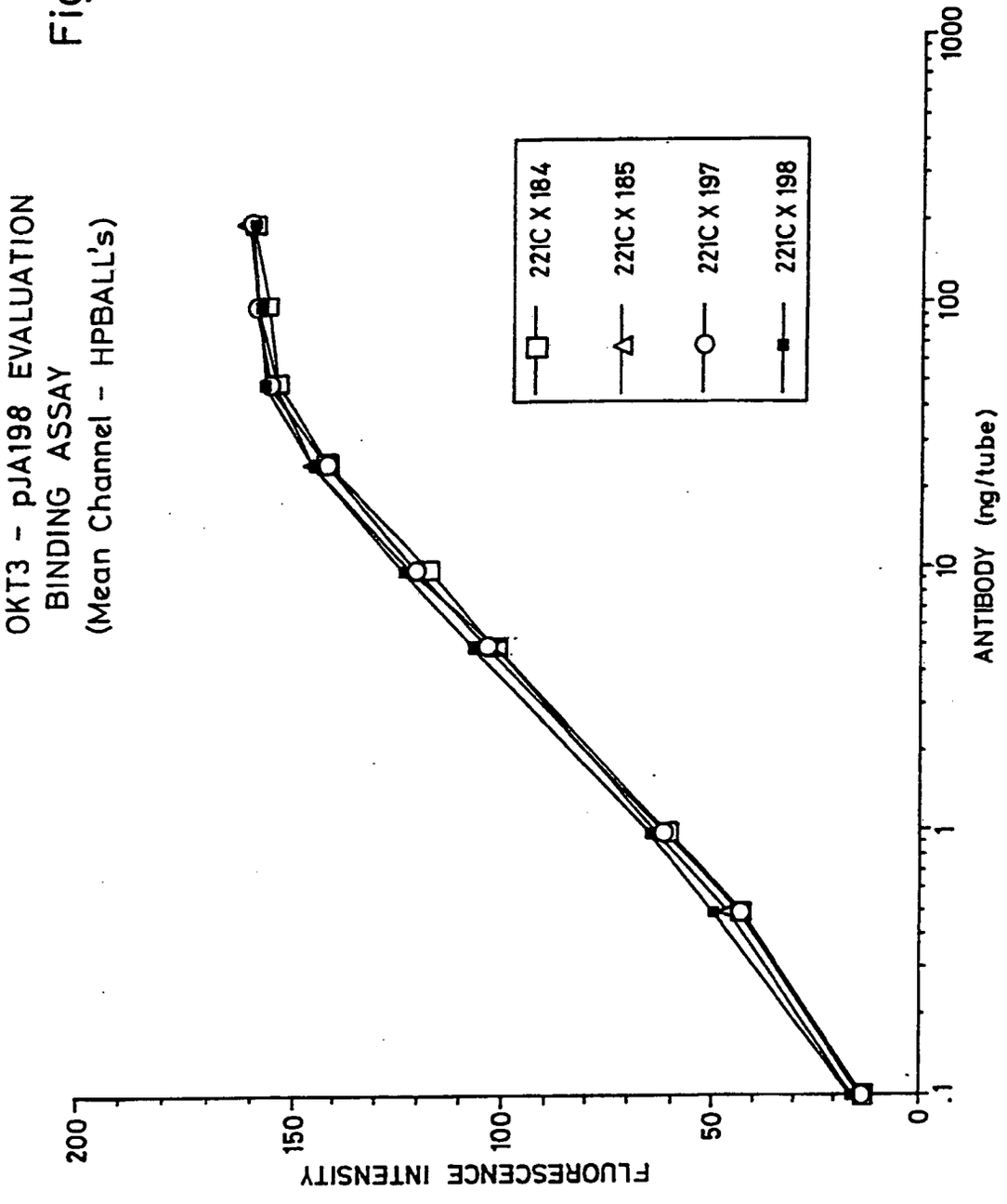
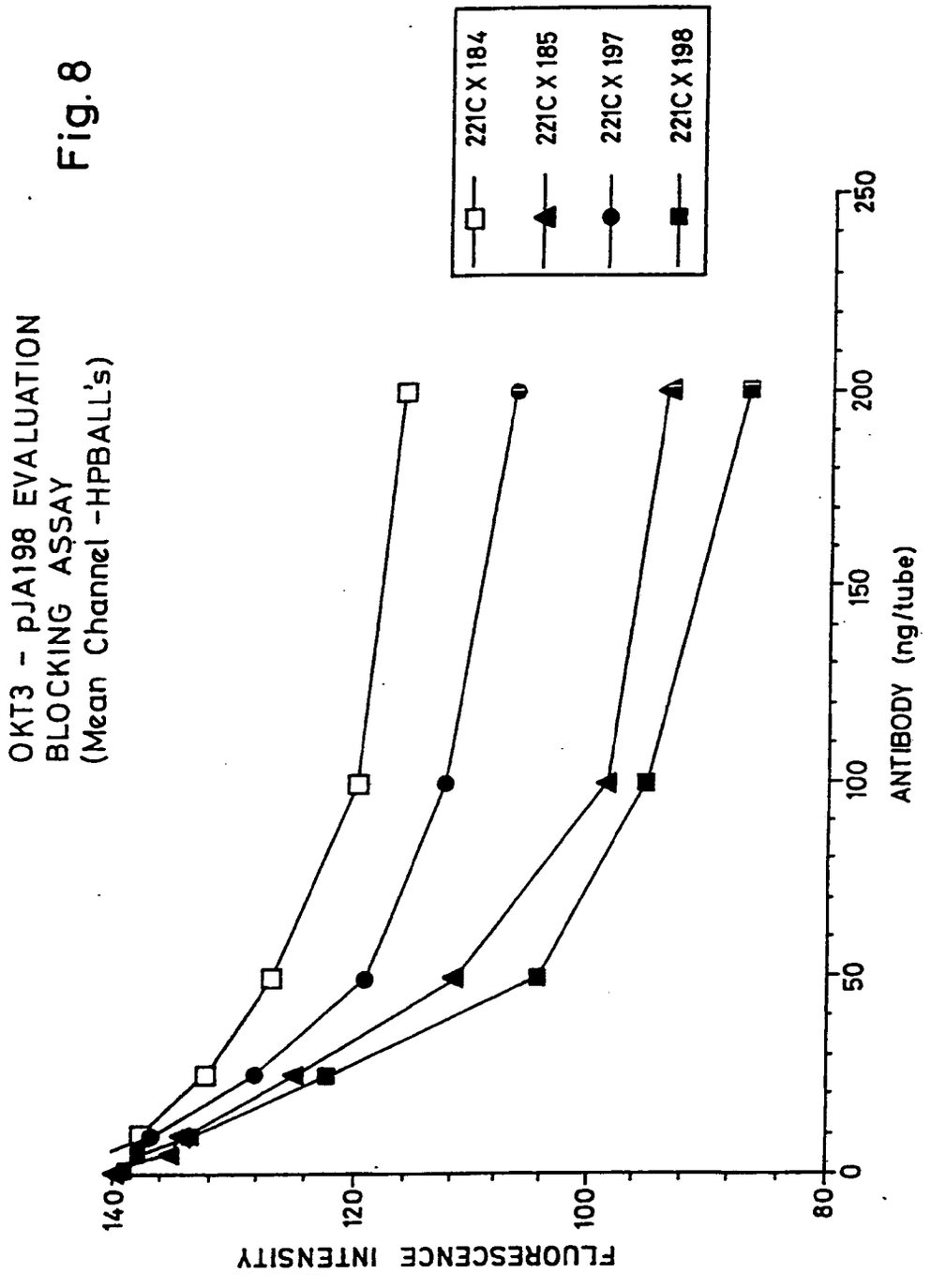


Fig. 8



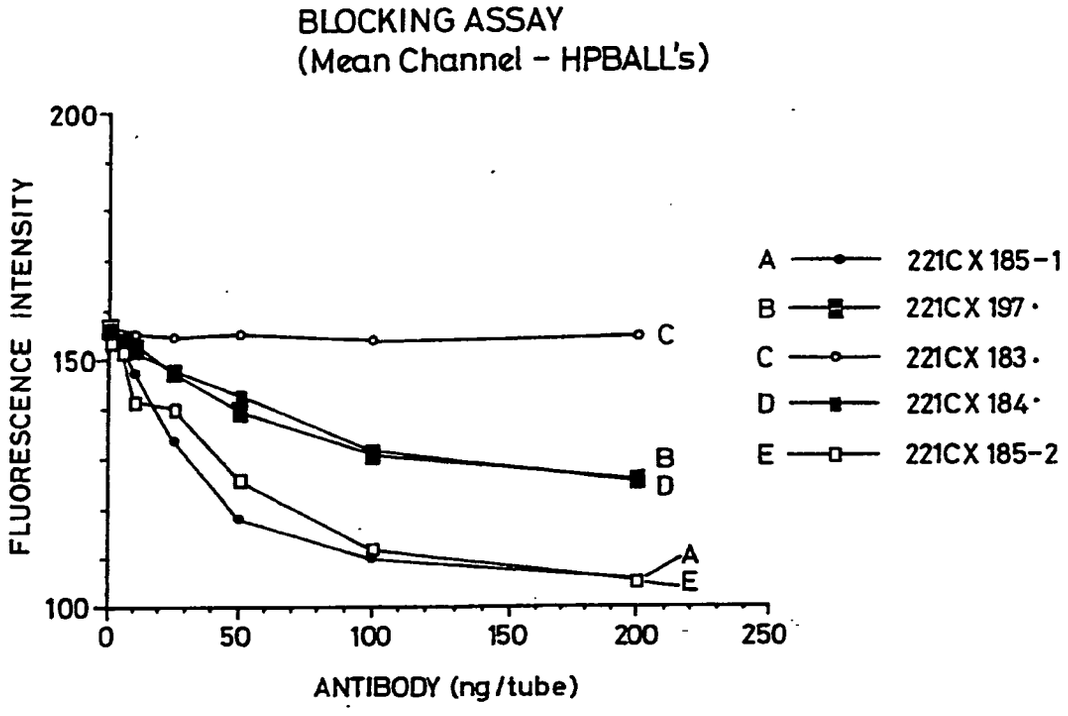
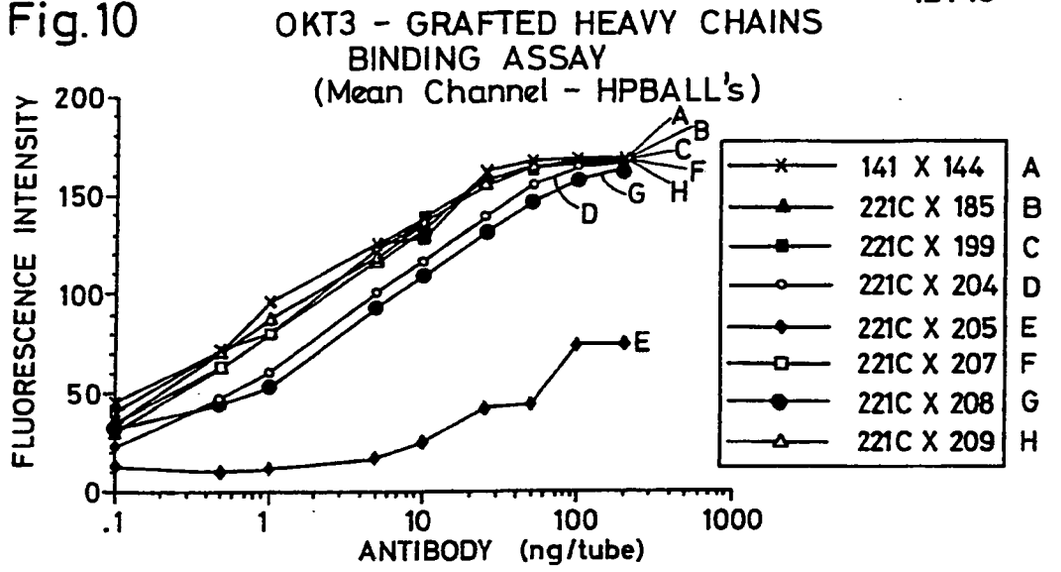
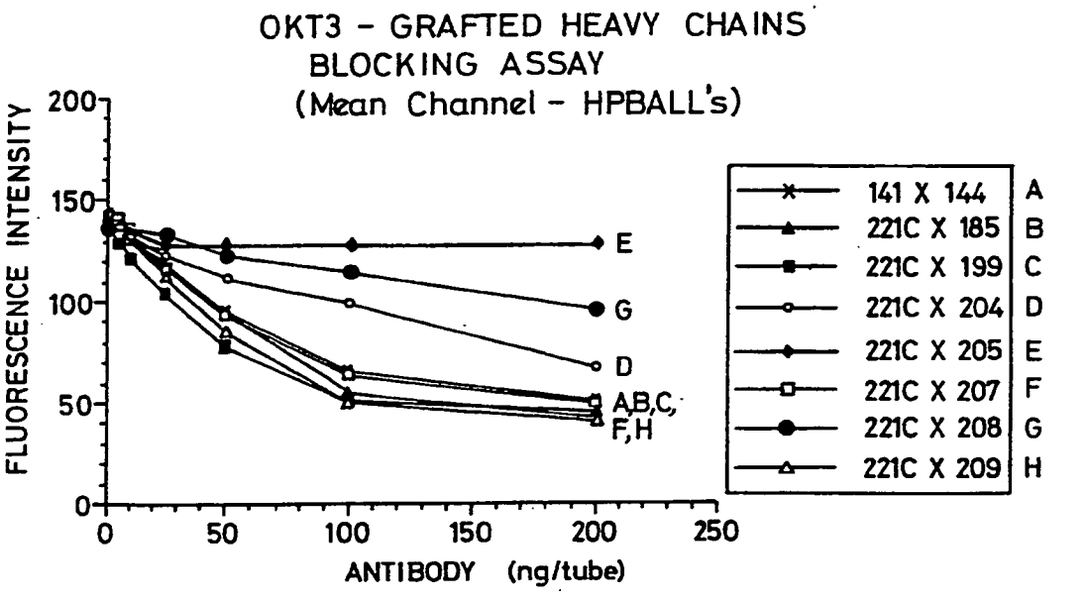


Fig. 9

Fig.10





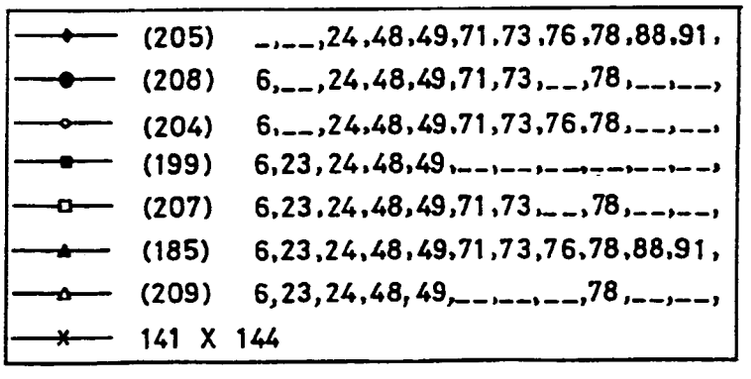
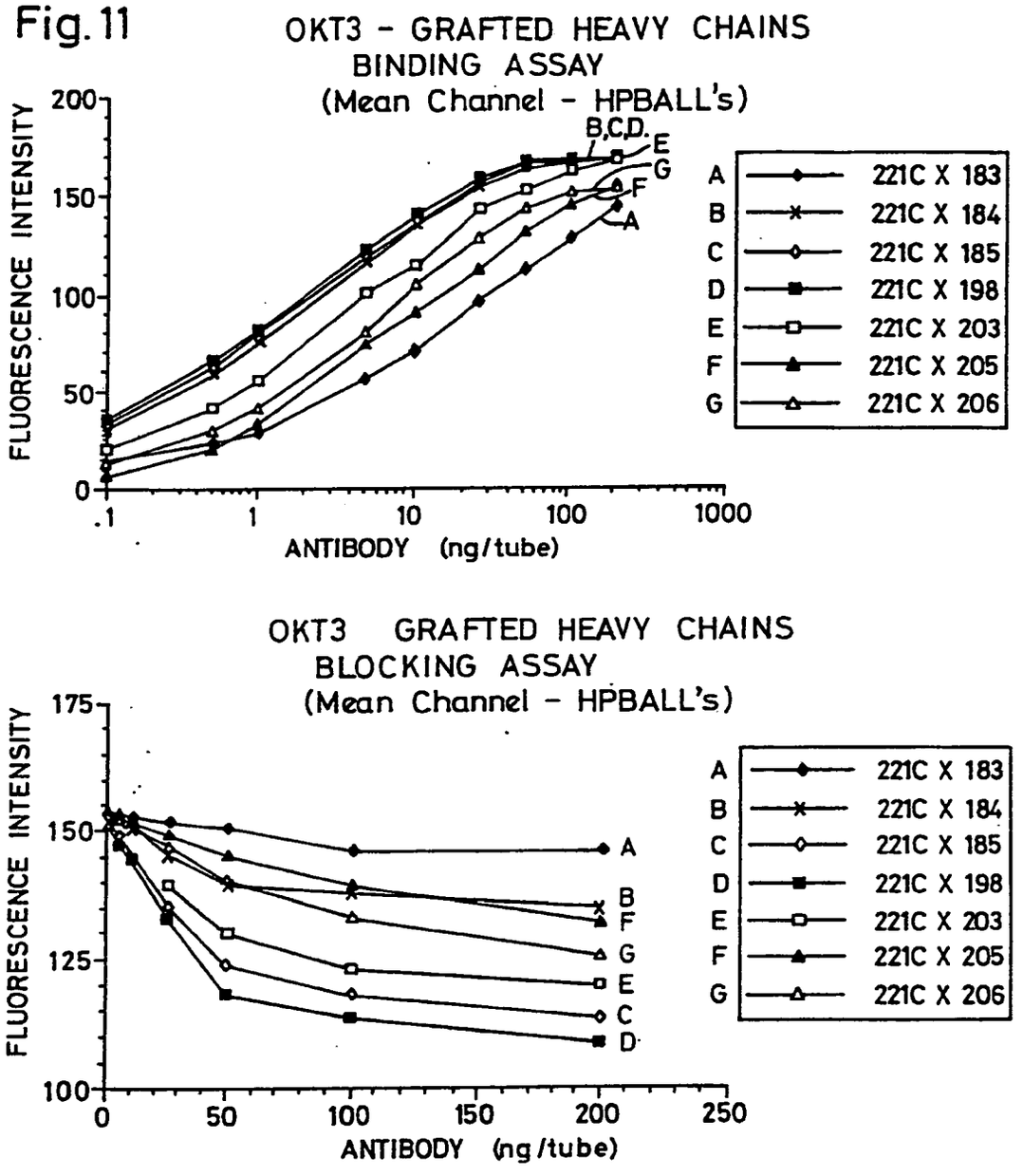


Fig. 11



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▲	(205)	_,_,_,24,48,49,71,73,76,78,88,91,
×	(184)	6,23,24,_,_,_,_,_,_,_,_,_,_,_,
△	(206)	_,_,_,24,48,49,71,73,76,78,_,_,_,
□	(203)	6,_,_,24,48,49,71,73,76,78,88,91,
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Fig. 12

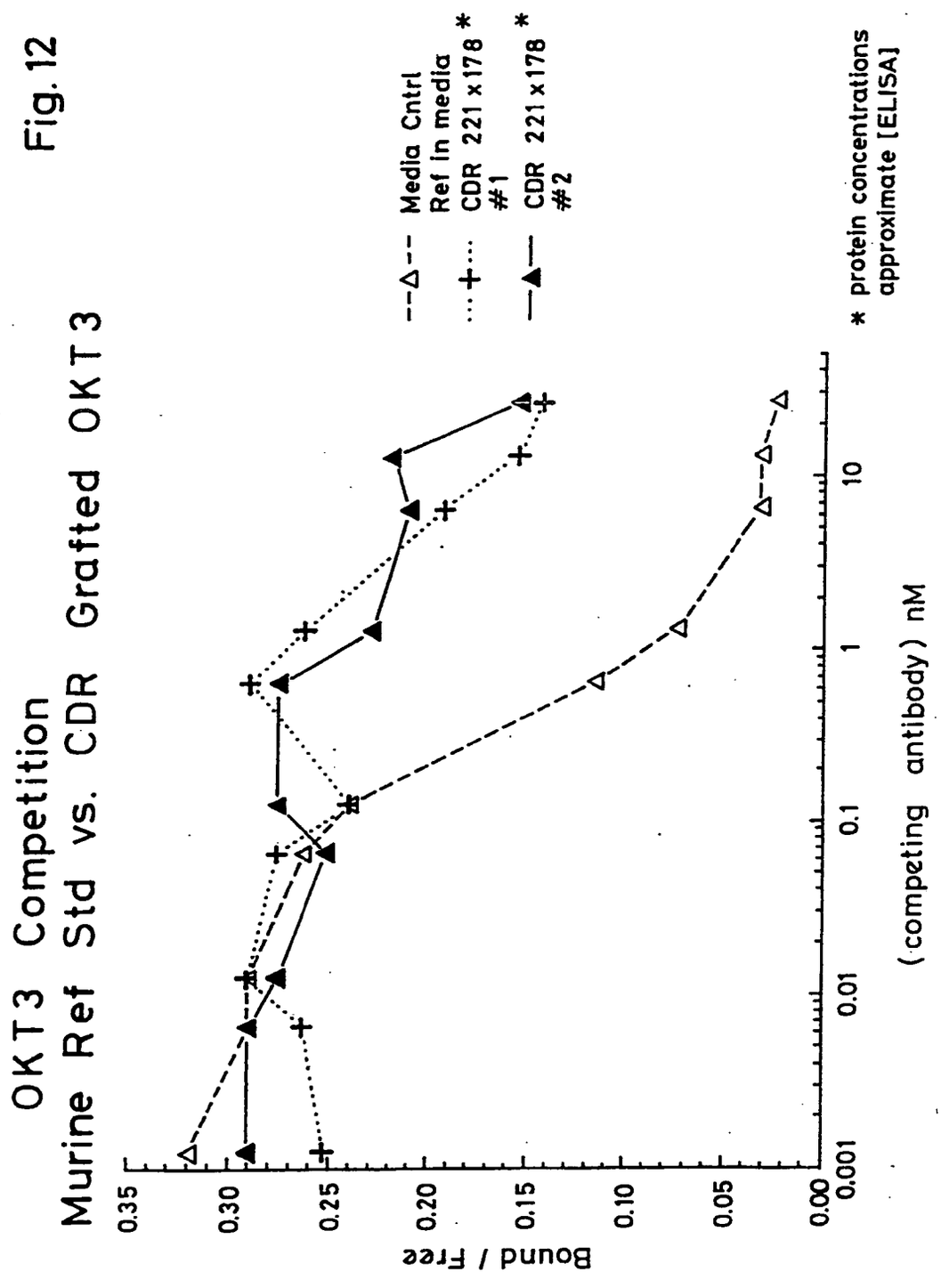
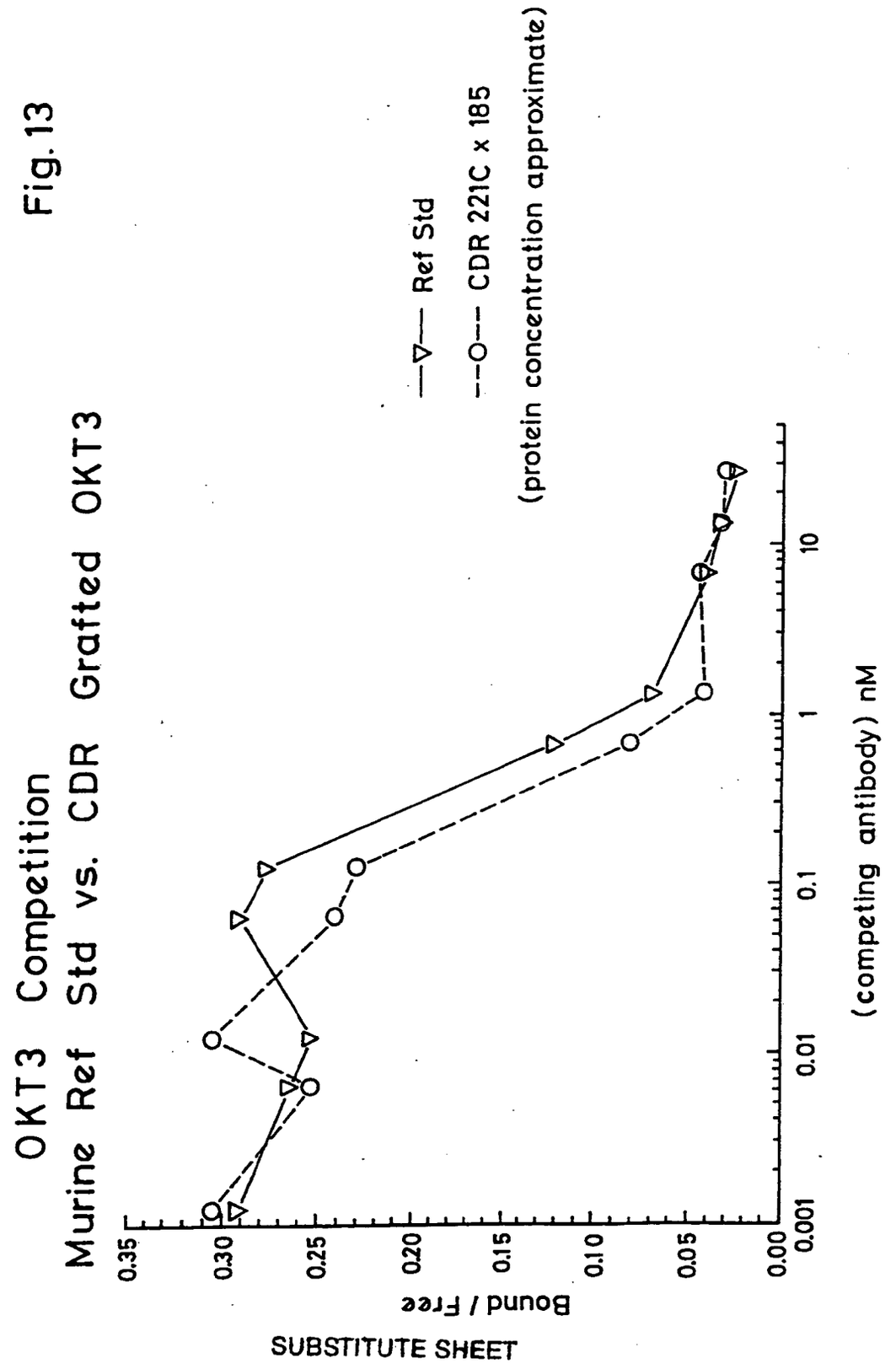


Fig. 13



INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 90/02017

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 12 P 21/08, C 12 N 15/13, A 61 K 39/395, C 07 K 15/06
C 12 N 5/10, 15/62

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	C 12 P; C 12 N; A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
P,X	EP, A1, 0403156 (GENZYME CORPORATION ET AL.) 19 December 1990, see examples 8-12 and corresponding tables	1,6,8, 13,14-22
Y	Proc. Natl. Acad. Sci. USA, vol. 86, December 1989, C. Queen et al.: "A humanized antibody that binds to the interleukin 2 receptor ", see page 10029- page 10033 see the whole document and in particular page 10031 right col. - page 10032, left col. and page 10033 left col.	1,6,8, 13,14-22
Y	EP, A1, 0328404 (MEDICAL RESEARCH COUNCIL ET AL.) 16 August 1989, see pages 1-3, page 9, lines 49-54 and the claims	1,6,8, 13,14-22

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
11th April 1991	17.05.91
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	F.W. HECK

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Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Nature, v l. 332, March 1988, L. Riechmann et al.: "Reshaping human antibodies for therapy ", see page 323 - page 327 see in particular page 327, right col.	1,6,8, 13,144-22
A	Nature, vol. 321, May 1986, P.T. Jones et al.: "Replacing the complementarity-determining regions in a human antibody with those from a mouse ", see page 522 - page 525 see the whole document	1-22
A	Nature, vol. 328, August 1987, S. Roberts et al.: "Generation of an antibody with enhanced affinity and specificity for its antigen by protein engineering ", see page 731 - page. 734 see the whole document	1,6
A	Science, vol. 239, 1988, M. Verhoeyen et al.: "Reshaping Human Antibodies: Grafting an Antilysozyme Activity ", see page 1534 - page 1536 see the whole document	1,6
A	EP, A2, 0239400 (WINTER, GREGORY PAUL) 30 September 1987, see the whole document	1,6,17-22
A	EP, A1, 0323806 (CIBA-GEIGY AG) 12 July 1989, see pages 2-6	1,6,17-22
A	Nature, vol. 341, October 1989, E.S. Ward et al.: "Binding activities of a repertoire of single immunoglobulin variable domains secreted from Escherichia coli ", see page 544 - page 546	1,6

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. PCT/GB 90/02017

SA 43080

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		WO-A-	89/07452	24/08/89
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		JP-A-	62296890	24/12/87
EP-A1- 0323806	12/07/89	AU-D-	2759588	06/07/89
		JP-A-	2154696	14/06/90

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(72) Inventors: FERRARA, Napoleone ; 3835 Scott, #306, San Francisco, CA 94123 (US). KIM, Kyung, Jin ; 94 Eastwood Drive, San Francisco, CA 94112 (US).

(74) Agents: JOHNSTON, Sean, A. et al.; Genentech, Inc., 460 Point San Bruno Boulevard, South San Francisco, CA 94080-4990 (US).

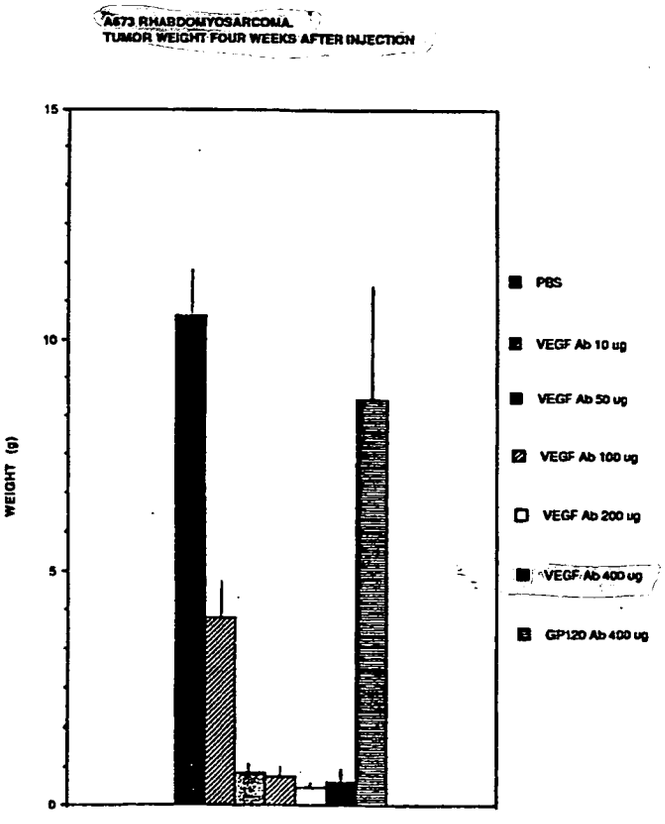
(81) Designated States: AU, BB, BG, BR, CA, CS, FI, HU, JP, KP, KR, LK, MG, MN, MW, NO, PL, RO, RU, SD, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, SN, TD, TG).

Published
With international search report.

(54) Title: VASCULAR ENDOTHELIAL CELL GROWTH FACTOR ANTAGONISTS

(57) Abstract

The present invention provides vascular endothelial cell growth factor (hVEGF) antagonists, including monoclonal antibodies, hVEGF receptors, and hVEGF variants that inhibit the mitogenic, angiogenic, or other biological activity of hVEGF. The antagonists thus are useful for the treatment of diseases and disorders characterized by undesirable or excessive endothelial cell proliferation or neovascularization. The monoclonal antibodies and receptors of the invention are also useful in diagnostic and analytical methods for determining the presence of hVEGF in a test sample.



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VASCULAR ENDOTHELIAL CELL GROWTH FACTOR ANTAGONISTS

Field of the Invention

The present invention relates to vascular endothelial cell growth factor (VEGF) antagonists, to therapeutic compositions comprising the antagonists, and to methods of use of the antagonists for diagnostic and therapeutic purposes.

Background of the Invention

The two major cellular components of the vasculature are the endothelial and smooth muscle cells. The endothelial cells form the lining of the inner surface of all blood vessels, and constitute a nonthrombogenic interface between blood and tissue. In addition, endothelial cells are an important component for the development of new capillaries and blood vessels. Thus, endothelial cells proliferate during the angiogenesis, or neovascularization, associated with tumor growth and metastasis, and a variety of non-neoplastic diseases or disorders.

Various naturally occurring polypeptides reportedly induce the proliferation of endothelial cells. Among those polypeptides are the basic and acidic fibroblast growth factors (FGF), Burgess and Maciag, Annual Rev. Biochem., 58:575 (1989), platelet-derived endothelial cell growth factor (PD-ECGF), Ishikawa, et al., Nature, 338:557 (1989), and vascular endothelial growth factor (VEGF), Leung, et al., Science 246:1306 (1989); Ferrara & Henzel, Biochem. Biophys. Res. Commun. 161:851 (1989); Tischer, et al., Biochem. Biophys. Res. Commun. 165:1198 (1989); Ferrara, et al., PCT Pat. Pub. No. WO 90/13649 (published November 15, 1990); Ferrara, et al., U.S. Pat. App. No. 07/360,229.

VEGF was first identified in media conditioned by bovine pituitary follicular or folliculostellate cells. Biochemical analyses indicate that bovine VEGF is a dimeric protein with an apparent molecular mass of approximately 45,000 Daltons, and with an apparent mitogenic specificity for vascular endothelial cells. DNA encoding bovine VEGF was isolated by screening a cDNA library prepared from such cells, using oligonucleotides based on the amino-terminal amino acid sequence of the protein as hybridization probes.

Human VEGF was obtained by first screening a cDNA library prepared from human cells, using bovine VEGF cDNA as a hybridization probe. One cDNA identified thereby encodes a 165-amino acid protein having greater than 95% homology to bovine VEGF, which protein is referred to as human VEGF (hVEGF). The mitogenic activity of human VEGF was confirmed by expressing the human VEGF cDNA in mammalian host cells. Media conditioned by cells transfected with the human VEGF cDNA promoted the proliferation of capillary endothelial cells, whereas control cells did not. Leung, et al., Science 246:1306 (1989).

Several additional cDNAs were identified in human cDNA libraries that encode 121-, 189-, and 206-amino acid isoforms of hVEGF (also collectively referred to as hVEGF-related proteins). The 121-amino acid protein differs from hVEGF by virtue of the deletion of the 44 amino acids between residues 116 and 159 in hVEGF. The 189-amino acid protein differs

from hVEGF by virtue of the ins rtion of 24 amino acids at residue 116 in hVEGF, and apparently is identical to human vascular permeability factor (hVPF). The 206-amino acid protein differs from hVEGF by virtue of an insertion of 41 amino acids at residue 116 in hVEGF. Houck, et al., Mol. Endocrin. 5:1806 (1991); Ferrara, et al., J. Cell. Biochem. 47:211 (1991); Ferrara, et al., Endocrine Reviews 13:18 (1992); Keck, et al., Science 246:1309 (1989); Connolly, et al., J. Biol. Chem. 264:20017 (1989); Keck, et al., EPO Pat. Pub. No. 0 370 989 (published May 30, 1990).

VEGF not only stimulates vascular endothelial cell proliferation, but also induces vascular permeability and angiogenesis. Angiogenesis, which involves the formation of new blood vessels from preexisting endothelium, is an important component of a variety of diseases and disorders including tumor growth and metastasis, rheumatoid arthritis, psoriasis, atherosclerosis, diabetic retinopathy, retrolental fibroplasia, neovascular glaucoma, hemangiomas, immune rejection of transplanted corneal tissue and other tissues, and chronic inflammation.

In the case of tumor growth, angiogenesis appears to be crucial for the transition from hyperplasia to neoplasia, and for providing nourishment to the growing solid tumor. Folkman, et al., Nature 339:58 (1989). Angiogenesis also allows tumors to be in contact with the vascular bed of the host, which may provide a route for metastasis of the tumor cells. Evidence for the role of angiogenesis in tumor metastasis is provided, for example, by studies showing a correlation between the number and density of microvessels in histologic sections of invasive human breast carcinoma and actual presence of distant metastases. Weidner, et al., New Engl. J. Med. 324:1 (1991).

In view of the role of vascular endothelial cell growth and angiogenesis, and the role of those processes in many diseases and disorders, it is desirable to have a means of reducing or inhibiting one or more of the biological effects of VEGF. It is also desirable to have a means of assaying for the presence of VEGF in normal and pathological conditions, and especially cancer.

Summary of the Invention

The present invention provides antagonists of VEGF, including (a) antibodies and variants thereof which are capable of specifically binding to hVEGF, hVEGF receptor, or a complex comprising hVEGF in association with hVEGF receptor, (b) hVEGF receptor and variants thereof, and (c) hVEGF variants. The antagonists inhibit the mitogenic, angiogenic, or other biological activity of hVEGF, and thus are useful for the treatment of diseases or disorders characterized by undesirable excessive neovascularization, including by way of example tumors, and especially solid malignant tumors, rheumatoid arthritis, psoriasis, atherosclerosis, diabetic and other retinopathies, retrolental fibroplasia, neovascular glaucoma, hemangiomas, thyroid hyperplasias (including Grave's disease), corneal and other tissue transplantation, and chronic inflammation. The antagonists also are useful for th treatment

of diseases or disorders characterized by undesirable excessive vascular permeability, such as edema associated with brain tumors, ascites associated with malignancies, Meigs' syndrome, lung inflammation, nephrotic syndrome, pericardial ffusion (such as that associated with pericarditis), and pleural effusion.

In other aspects, the VEGF antagonists are polyspecific monoclonal antibodies which are capable of binding to (a) a non-hVEGF epitope, for example, an epitope of a protein involved in thrombogenesis or thrombolysis, or a tumor cell surface antigen, and to (b) hVEGF, hVEGF receptor, or a complex comprising hVEGF in association with hVEGF receptor.

In still other aspects, the VEGF antagonists are conjugated with a cytotoxic moiety.

In another aspect, the invention concerns isolated nucleic acids encoding the monoclonal antibodies as hereinbefore described, and hybridoma cell lines which produce such monoclonal antibodies.

In another aspect, the invention concerns pharmaceutical compositions comprising a VEGF antagonist in an amount effective in reducing or eliminating hVEGF-mediated mitogenic or angiogenic activity in a mammal.

In a different aspect, the invention concerns methods of treatment comprising administering to a mammal, preferably a human patient in need of such treatment, a physiologically effective amount of a VEGF antagonist. If desired, the VEGF antagonist is coadministered, either simultaneously or sequentially, with one or more other VEGF antagonists or anti-tumor or anti-angiogenic substances.

In another aspect, the invention concerns a method for detecting hVEGF in a test sample by means of contacting the test sample with an antibody capable of binding specifically to hVEGF and determining the extent of such binding.

Brief Description of the Drawings

Figure 1 shows the effect of anti-hVEGF monoclonal antibodies (A4.6.1 or B2.6.2) or an irrelevant anti-hepatocyte growth factor antibody (anti-HGF) on the binding of the anti-hVEGF monoclonal antibodies to hVEGF.

Figure 2 shows the effect of anti-hVEGF monoclonal antibodies (A4.6.1 or B2.6.2) or an irrelevant anti-HGF antibody on the biological activity of hVEGF in cultures of bovine adrenal cortex capillary endothelial (ACE) cells.

Figure 3 shows the effect of anti-hVEGF monoclonal antibodies (A4.6.1, B2.6.2, or A2.6.1) on the binding of hVEGF to bovine ACE cells.

Figure 4 shows the effect of A4.6.1 anti-hVEGF monoclonal antibody treatment on the rate of growth of growth of NEG55 tumors in mice.

Figure 5 shows the effect of A4.6.1 anti-hVEGF monoclonal antibody treatment on the size of NEG55 tumors in mice after five weeks of treatment.

Figure 6 shows the effect of A4.6.1 anti-hVEGF monoclonal antibody (VEGF Ab) treatment on the growth of SK-LMS-1 tumors in mice.

Figure 7 shows the effect of varying doses of A4.6.1 anti-hVEGF monoclonal antibody (VEGF Ab) treatment on the growth of A673 tumors in mic . is shown in

Figure 8 shows the effect of A4.6.1 anti-hVEGF monoclonal antibody on the growth and survival of NEG55 (G55) glioblastoma cells in culture.

Figure 9 shows the effect of A4.6.1 anti-hVEGF monoclonal antibody on the growth and survival of A673 rhabdomyosarcoma cells in culture.

Figure 10 shows the effect of A4.6.1 anti-hVEGF monoclonal antibody on human synovial fluid-induced chemotaxis of human endothelial cells.

Detailed Description of the Invention

The term "hVEGF" as used herein refers to the 165-amino acid human vascular endothelial cell growth factor, and related 121-, 189-, and 206-amino acid vascular endothelial cell growth factors, as described by Leung, et al., Science 246:1306 (1989), and Houck, et al., Mol. Endocrin. 5:1806 (1991) together with the naturally occurring allelic and processed forms of those growth factors.

The present invention provides antagonists of hVEGF which are capable of inhibiting one or more of the biological activities of hVEGF, for example, its mitogenic or angiogenic activity. Antagonists of hVEGF act by interfering with the binding of hVEGF to a cellular receptor, by incapacitating or killing cells which have been activated by hVEGF, or by interfering with vascular endothelial cell activation after hVEGF binding to a cellular receptor. All such points of intervention by an hVEGF antagonist shall be considered equivalent for purposes of this invention. Thus, included within the scope of the invention are antibodies, and preferably monoclonal antibodies, or fragments thereof, that bind to hVEGF, hVEGF receptor, or a complex comprising hVEGF in association with hVEGF receptor. Also included within the scope of the invention are fragments and amino acid sequence variants of hVEGF that bind to hVEGF receptor but which do not exhibit a biological activity of native hVEGF. Also included within the scope of the invention are hVEGF receptor and fragments and amino acid sequence variants thereof which are capable of binding hVEGF.

The term "hVEGF receptor" or "hVEGFr" as used herein refers to a cellular for hVEGF, ordinarily a cell-surface receptor found on vascular endothelial cells, as well as variants thereof which retain the ability to bind hVEGF. Typically, the hVEGF receptors and variants thereof that are hVEGF antagonists will be in isolated form, rather than being integrated into a cell membrane or fixed to a cell surface as may be the case in nature. One example of a hVEGF receptor is the fms-like tyrosine kinase (flt), a transmembrane receptor in the tyrosine kinase family. DeVries, et al., Science 255:989 (1992); Shibuya, et al., Oncogene 5:519 (1990). The flt receptor comprises an extracellular domain, a transmembrane domain, and an intracellular domain with tyrosine kinase activity. The extracellular domain is involved in the binding of hVEGF, whereas the intracellular domain is involved in signal transduction.

Another xample f an hVEGF receptor is the flk-1 receptor (also referred to as KDR). Matthews, et al., Proc. Nat. Acad. Sci. 88:9026 (1991); Terman, et al., Oncogen 6:1677 (1991); Terman, et al., Biochem. Biophys. Res. Commun. 187:1579 (1992).

Binding of hVEGF to the flt receptor results in th formation of at least two high molecular weight complexes, having apparent molecular weight of 205,000 and 300,000 Daltons. The 300,000 Dalton complex is believed to be a dimer comprising two receptor molecules bound to a single molecule of hVEGF.

Variants of hVEGFr also are included within the scope hereof. Representative examples include truncated forms of a receptor in which the transmembrane and cytoplasmic domains are deleted from the receptor, and fusions proteins in which non-hVEGFr polymers or polypeptides are conjugated to the hVEGFr or, preferably, truncated forms thereof. An example of such a non-hVEGF polypeptide is an immunoglobulin. In that case, for example, the extracellular domain of the hVEGFr is substituted for the Fv domain of an immunoglobulin light or (preferably) heavy chain, with the C-terminus of the receptor extracellular domain covalently joined to the amino terminus of the CH1, hinge, CH2 or other fragment of the heavy chain. Such variants are made in the same fashion as known immunoadhesons. See e.g., Gascoigne, et al., Proc. Nat. Acad. Sci. 84:2936 (1987); Capon, et al., Nature 337:525 (1989); Aruffo, et al., Cell 61:1303 (1990); Ashkenazi, et al., Proc. Nat. Acad. Sci. 88:10535 (1991); Bennett, et al., J. Biol. Chem. 266:23060 (1991). In other embodiments, the hVEGFr is conjugated to a non-proteinaceous polymer such as polyethylene glycol (PEG) (see e.g., Davis, et al., U.S. Patent No. 4,179,337; Goodson, et al., BioTechnology 8:343-346 (1990); Abuchowski, et al., J. Biol. Chem. 252:3578 (1977); Abuchowski, et al., J. Biol. Chem. 252:3582 (1977)) or carbohydrates (see e.g., Marshall, et al., Arch. Biochem. Biophys., 167:77 (1975)). This serves to extend the biological half-life of the hVEGFr and reduces the possibility that the receptor will be immunogenic in the mammal to which it is administered. The hVEGFr is used in substantially the same fashion as antibodies to hVEGF, taking into account the affinity of the antagonist and its valency for hVEGF.

The extracellular domain of hVEGF receptor, either by itself or fused to an immunoglobulin polypeptide or other carrier polypeptide, is especially useful as an antagonist of hVEGF, by virtue of its ability to sequester hVEGF that is present in a host but that is not bound to hVEGFr on a cell surface.

hVEGFr and variants thereof also are useful in screening assays to identify agonists and antagonists of hVEGF. For example, host cells transfected with DNA encoding hVEGFr (for example, flt or flk1) overexpress the receptor polypeptide on the cell surface, making such recombinant host cells ideally suited for analyzing the ability of a test compound (for example, a small molecule, linear or cyclic peptide, or polypeptide) to bind to hVEGFr. hVEGFr and hVEGFr fusion proteins, such as an hVEGFr-IgG fusion protein, may be used in a similar fashion. For example, the fusion protein is bound to an immobilized support and the ability

of a test compound t displace radiolabeled hVEGF from the hVEGFr domain of th fusion protein is determined.

The term "recombinant" used in reference to hVEGF, hVEGF receptor, monoclonal antibodies, or other proteins, refers to proteins that are produced by recombinant DNA expression in a host cell. The host cell may be prokaryotic (for example, a bacterial cell such as E. coli) or eukaryotic (for example, a yeast or a mammalian cell).

Antagonist Monoclonal Antibodies

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical in specificity and affinity except for possible naturally occurring mutations that may be present in minor amounts. It should be appreciated that as a result of such naturally occurring mutations and the like, a monoclonal antibody composition of the invention, which will predominantly contain antibodies capable of specifically binding hVEGF, hVEGFr, or a complex comprising hVEGF in association with hVEGFr ("hVEGF-hVEGFr complex"), may also contain minor amounts of other antibodies.

Thus, the modifier "monoclonal" indicates the character of the antibody as being obtained from such a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, monoclonal antibodies of the invention may be made using the hybridoma method first described by Kohler & Milstein, Nature 256:495 (1975), or may be made by recombinant DNA methods. Cabilly, et al., U.S. Pat. No. 4,816,567.

In the hybridoma method, a mouse or other appropriate host animal is immunized with antigen by subcutaneous, intraperitoneal, or intramuscular routes to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein(s) used for immunization. Alternatively, lymphocytes may be immunized in vitro. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell. Goding, Monoclonal Antibodies: Principles and Practice, pp.59-103 (Academic Press, 1986).

The antigen may be hVEGF, hVEGFr, or hVEGF-hVEGFr complex. The antigen optionally is a fragment or portion of any one of hVEGF or hVEGFr having one or more amino acid residues that participate in the binding of hVEGF to one of its receptors. For example, immunization with the extracellular domain of an hVEGFr (that is, a truncated hVEGFr polypeptide lacking transmembrane and intracellular domains) will be especially useful in producing antibodies that are antagonists of hVEGF, since it is the extracellular domain that is involved in hVEGF binding.

Monoclonal antibodies capable of binding hVEGF-hVEGFr complex are useful, particularly if they do not also bind to non-associated (non-complexed) hVEGF and hVEGFr. Such antibodies thus only bind to cells undergoing immediate activation by hVEGF and

accordingly are not sequestered by free hVEGF or hVEGFr as is normally found in a mammal. Such antibodies typically bind an epitope that spans one or more points of contact between the receptor and hVEGF. Such antibodies have been produced for other ligand receptor complexes and may be produced here in the same fashion. These antibodies need not, and may not, neutralize or inhibit a biological activity of non-associated hVEGF or hVEGFr, whether or not the antibodies are capable of binding to non-associated hVEGF or hVEGFr.

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high level expression of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, California USA, SP-2 cells available from the American Type Culture Collection, Rockville, Maryland USA, and P3X63Ag8U.1 cells described by Yelton, et al., Curr. Top. Microbiol. Immunol. 81:1 (1978). Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies. Kozbor, J. Immunol. 133:3001 (1984). Brodeur, et al., Monoclonal Antibody Production Techniques and Applications, pp.51-63 (Marcel Dekker, Inc., New York, 1987).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA). The monoclonal antibodies of the invention are those that preferentially immunoprecipitate hVEGF, hVEGFr, or hVEGF-hVEGFr complex, or that preferentially bind to at least one of those antigens in a binding assay, and that are capable of inhibiting a biological activity of hVEGF.

After hybridoma cells are identified that produce antagonist antibodies of the desired specificity, affinity, and activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods. Goding, Monoclonal Antibodies: Principles and Practice, pp.59-104 (Academic Press, 1986). Suitable culture media for this purpose include, for example, Dulbecco's Modified Eagle's Medium or RPMI-1640 medium. In addition, the hybridoma cells may be grown in vivo as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascit s fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

DNA encoding the monoclonal antibodies of the invention is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells of the invention serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as simian COS cells, Chinese Hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells.

The DNA optionally may be modified in order to change the character of the immunoglobulin produced by its expression. For example, humanized forms of murine antibodies are produced by substituting a complementarity determining region (CDR) of the murine antibody variable domain for the corresponding region of a human antibody. In some embodiments, selected framework region (FR) amino acid residues of the murine antibody also are substituted for the corresponding amino acid residues in the human antibody. Carter, et al., Proc. Nat. Acad. Sci. 89:4285 (1992); Carter, et al., BioTechnology 10:163 (1992). Chimeric forms of murine antibodies also are produced by substituting the coding sequence for selected human heavy and light constant chain domains in place of the homologous murine sequences. Cabilly, et al., U.S. Pat. No. 4,816,567; Morrison, et al., Proc. Nat. Acad. Sci. 81:6851 (1984).

The antibodies included within the scope of the invention include variant antibodies, such as chimeric (including "humanized") antibodies and hybrid antibodies comprising immunoglobulin chains capable of binding hVEGF, hVEGFr, or hVEGF-hVEGFr complex, and a non-hVEGF epitope.

The antibodies herein include all species of origin, and immunoglobulin classes (e.g., IgA, IgD, IgE, IgG, and IgM) and subclasses, as well as antibody fragments (e.g., Fab, $F(ab')_2$, and Fv), so long as they are capable of binding hVEGF, hVEGFr, or hVEGF-hVEGFr complex, and are capable of antagonizing a biological activity of hVEGF.

In a preferred embodiment of the invention, the monoclonal antibody will have an affinity for the immunizing antigen of at least about 10^9 liters/mole, as determined, for example, by the Scatchard analysis of Munson & Pollard, Anal. Biochem. 107:220 (1980). Also, the monoclonal antibody typically will inhibit the mitogenic or angiogenic activity of hVEGF at least about 50%, preferably greater than 80%, and most preferably greater than 90%, as determined, for example, by an in vitro cell survival or proliferation assay, such as described in Example 2.

For some therapeutic and diagnostic applications, it is desirable that the monoclonal antibody be reactive with fewer than all of the different molecular forms of hVEGF. For example, it may be desirable to have a monoclonal antibody that is capable of specifically binding to the 165-amino acid sequence hVEGF but not to the 121- or 189-amino acid sequence hVEGF polypeptides. Such antibodies are readily identified by comparative ELISA assays or comparative immunoprecipitation of the different hVEGF polypeptides.

Conjugates with Cytotoxic Moieties

In some embodiments it is desireable to provide a cytotoxic moiety conjugated to a hVEGF-specific monoclonal antibody or to hVEGFr. In these embodiments the cytotoxin serves to incapacitate or kill cells which are expressing or binding hVEGF or its receptor. The conjugate is targeted to the cell by the domain which is capable of binding to hVEGF, hVEGFr, or hVEGF-hVEGFr complex. Thus, monoclonal antibodies that are capable of binding hVEGF, hVEGFr, or hVEGF-hVEGFr complex are conjugated to cytotoxins. Similarly, hVEGFr is conjugated to a cytotoxin. While the monoclonal antibodies optimally are capable of neutralizing the activity of hVEGF alone (without the cytotoxin), it is not necessary in this embodiment that the monoclonal antibody or receptor be capable of any more than binding to hVEGF, hVEGFr, or hVEGF-hVEGFr complex.

Typically, the cytotoxin is a protein cytotoxin, e.g. diptheria, ricin or Pseudomonas toxin, although in the case of certain classes of immunoglobulins the Fc domain of the monoclonal antibody itself may serve to provide the cytotoxin (e.g., in the case of IgG2 antibodies, which are capable of fixing complement and participating in antibody-dependent cellular cytotoxicity (ADCC)). However, the cytotoxin does not need to be proteinaceous and may include chemotherapeutic agents heretofore employed, for example, for the treatment of tumors.

The cytotoxin typically is linked to a monoclonal antibody or fragment thereof by a backbone amide bond within (or in place of part or all of) the Fc domain of the antibody. Where the targeting function is supplied by hVEGFr, the cytotoxic moiety is substituted onto any domain of the receptor that does not participate in hVEGF binding; preferably, the moiety is substituted in place of or onto the transmembrane and or cytoplasmic domains of the receptor. The optimal site of substitution will be determined by routine experimentation and is well within the ordinary skill.

Conjugates which are protein fusions are easily made in recombinant cell culture by expressing a gene encoding the conjugate. Alternatively, the conjugates are made by covalently crosslinking the cytotoxic moiety to an amino acid residue side chain or C-terminal carboxyl of the antibody or the receptor, using methods known per se such as disulfide exchange or linkage through a thioester bond using for example iminothiolate and methyl-4-mercaptobutyrimadate.

Conjugates with other Moieti s

Th monoclonal antibodies and hVEGFr that are antagonists of hVEGF also are conjugated to substances that may not be readily classified as cytotoxins in their own right, but which augment the activity of the compositions herein. For example, monoclonal antibodies or hVEGFr capable of binding to hVEGF, hVEGFr, or hVEGF-hVEGFr complex are fused with heterologous polypeptides, such as viral sequences, with cellular receptors, with cytokines such as TNF, interferons, or interleukins, with polypeptides having procoagulant activity, and with other biologically or immunologically active polypeptides. Such fusions are readily made by recombinant methods. Typically such non-immunoglobulin polypeptides are substituted for the constant domain(s) of an anti-hVEGF or anti-hVEGF-hVEGFr complex antibody, or for the transmembrane and/or intracellular domain of an hVEGFr. Alternatively, they are substituted for a variable domain of one antigen binding site of an anti-hVEGF antibody described herein.

In preferred embodiments, such non-immunoglobulin polypeptides are joined to or substituted for the constant domains of an antibody described herein. Bennett, et al., J. Biol. Chem. 266:23060-23067 (1991). Alternatively, they are substituted for the Fv of an antibody herein to create a chimeric polyvalent antibody comprising at least one remaining antigen binding site having specificity for hVEGF, hVEGFr, or a hVEGF-hVEGFr complex, and a surrogate antigen binding site having a function or specificity distinct from that of the starting antibody.

Heterospecific Antibodies

Monoclonal antibodies capable of binding to hVEGF, hVEGFr, or hVEGF-hVEGFr complex need only contain a single binding site for the enumerated epitopes, typically a single heavy-light chain complex or fragment thereof. However, such antibodies optionally also bear antigen binding domains that are capable of binding an epitope not found within any one of hVEGF, hVEGFr, or hvEGF-hVEGFr complex. For example, substituting the corresponding amino acid sequence or amino acid residues of a native anti-hVEGF, anti-HVEGFr, or anti-hVEGF-hVEGFr complex antibody with the complementarity-determing and, if necessary, framework residues of an antibody having specificity for an antigen other than hVEGF, hVEGFr, or hVEGF-hVEGFr complex will create a polyspecific antibody comprising one antigen binding site having specificity for hVEGF, hVEGFr, or hVEGF-hVEGFr complex, and another antigen binding site having specificity for the non-hVEGF, hVEGFr, or hVEGF-hVEGFr complex antigen. These antibodies are at least bivalent, but may be polyvalent, depending upon the number of antigen binding sites possessed by the antibody class chosen. For example, antibodies of the IgM class will be polyvalent.

In preferred embodiments of the invention such antibodies are capable of binding an hVEGF or hVEGFr epitope and either (a) a polypeptide active in blood coagulation, such as prot in C or tissue factor, (b) a cytotoxic protein such as tumor necrosis factor (TNF), or (c)

a non-hVEGFr cell surface receptor, such as CD4, or HER-2 receptor (Maddon, et al., Cell 42:93 (1985); Coussens, et al., Science 230:1137 (1985)). Heterospecific, multivalent antibodies are conveniently made by cotransforming a host cell with DNA encoding the heavy and light chains of both antibodies and thereafter recovering, by immunoaffinity chromatography or the like, the proportion of expressed antibodies having the desired antigen binding properties. Alternatively, such antibodies are made by in vitro recombination of monospecific antibodies.

Monovalent Antibodies

Monovalent antibodies capable of binding to hVEGFr or hVEGF-hVEGFr complex are especially useful as antagonists of hVEGF. Without limiting the invention to any particular mechanism of biological activity, it is believed that activation of cellular hVEGF receptors proceeds by a mechanism wherein the binding of hVEGF to cellular hVEGF receptors induces aggregation of the receptors, and in turn activates intracellular receptor kinase activity. Because monovalent anti-hVEGF receptor antibodies cannot induce such aggregation, and therefore cannot activate hVEGF receptor by that mechanism, they are ideal antagonists of hVEGF.

It should be noted, however, that these antibodies should be directed against the hVEGF binding site of the receptor or should otherwise be capable of interfering with hVEGF binding to the receptor hVEGF, such as by sterically hindering hVEGF access to the receptor. As described elsewhere herein, however, anti-hVEGFr antibodies that are not capable of interfering with hVEGF binding are useful when conjugated to non-immunoglobulin moieties, for example, cytotoxins.

Methods for preparing monovalent antibodies are well known in the art. For example, one method involves recombinant expression of immunoglobulin light chain and modified heavy chain. The heavy chain is truncated generally at any point in the Fc region so as to prevent heavy chain crosslinking. Alternatively, the relevant cysteine residues are substituted with another amino acid residue or are deleted so as to prevent crosslinking. In vitro methods are also suitable for preparing monovalent antibodies. For example, Fab fragments are prepared by enzymatic cleavage of intact antibody.

Diagnostic Uses

For diagnostic applications, the antibodies or hVEGFr of the invention typically will be labeled with a detectable moiety. The detectable moiety can be any one which is capable of producing, either directly or indirectly, a detectable signal. For example, the detectable moiety may be a radioisotope, such as ^{3}H, ^{14}C, ^{32}P, ^{35}S, or ^{125}I, a fluorescent or chemiluminescent compound, such as fluorescein isothiocyanate, rhodamine, or luciferin; radioactive isotopic labels, such as, e.g., ^{125}I, ^{32}P, ^{14}C, or ^{3}H, or an enzyme, such as alkaline phosphatase, beta-galactosidase or horseradish peroxidase.

Any method known in the art for separately conjugating the antibody or hVEGFr to the detectable moiety may be employed, including those m thods described by Hunter, et al., Natur 144:945 (1962); David, et al., Biochemistry 13:1014 (1974); Pain, t al., J. Immunol. M th. 40:219 (1981); and Nygren, J. Histochem. and Cytochem. 30:407 (1982).

The antibodies and receptors of the present invention may be employed in any known assay method, such as competitive binding assays, direct and indirect sandwich assays, and immunoprecipitation assays. Zola, Monoclonal Antibodies: A Manual of Techniques, pp.147-158 (CRC Press, Inc., 1987).

Competitive binding assays rely on the ability of a labeled standard (which may be hVEGF or an immunologically reactive portion thereof) to compete with the test sample analyte (hVEGF) for binding with a limited amount of antibody. The amount of hVEGF in the test sample is inversely proportional to the amount of standard that becomes bound to the antibodies or receptors. To facilitate determining the amount of standard that becomes bound, the antibodies or receptors generally are insolubilized before or after the competition, so that the standard and analyte that are bound to the antibodies or receptors may conveniently be separated from the standard and analyte which remain unbound.

Sandwich assays involve the use of two antibodies or receptors, each capable of binding to a different immunogenic portion, or epitope, of the protein to be detected. In a sandwich assay, the test sample analyte is bound by a first antibody or receptor which is immobilized on a solid support, and thereafter a second antibody binds to the analyte, thus forming an insoluble three part complex. David & Greene, U.S. Pat No. 4,376,110. The second antibody or receptor may itself be labeled with a detectable moiety (direct sandwich assays) or may be measured using an anti-immunoglobulin antibody that is labeled with a detectable moiety (indirect sandwich assay). For example, one type of sandwich assay is an ELISA assay, in which case the detectable moiety is an enzyme.

The antibodies or receptor herein also is useful for in vivo imaging, wherein an antibody or hVEGFr labeled with a detectable moiety is administered to a patient, preferably into the bloodstream, and the presence and location of the labeled antibody or receptor in the patient is assayed. This imaging technique is useful, for example, in the staging and treatment of neoplasms. The antibody or hVEGFr is labeled with any moiety that is detectable in a mammal, whether by nuclear magnetic resonance, radiology, or other detection means known in the art.

Antagonist Variants of hVEGF

In addition to the antibodies described herein, other useful antagonists of hVEGF include fragments and amino acid sequence variants of native hVEGF that bind to hVEGF receptor but that do not exhibit the biological activity of native hVEGF. For example, such antagonists include fragments and amino acid sequence variants that comprise a receptor binding domain of hVEGF, but that lack a domain conferring biological activity, or that

otherwise are defective in activating cellular hVEGF receptors, such as in the case of a fragment or an amino acid sequence variant that is deficient in its ability to induce aggregation or activation of cellular hVEGF receptors. The term "receptor binding domain" refers to the amino acid sequences in hVEGF that are involved in hVEGF receptor binding. The term "biological activity domain" or "domain conferring biological activity" refers to an amino acid sequence in hVEGF that confer a particular biological activity of the factor, such as mitogenic or angiogenic activity.

The observation that hVEGF appears to be capable of forming a complex with two or more hVEGFr molecules on the surface of a cell suggests that hVEGF has at least two discrete sites for binding to hVEGFr and that it binds to such cellular receptors in sequential fashion, first at one site and then at the other before activation occurs, in the fashion of growth hormone, prolactin and the like (see e.g., Cunningham, et al., Science 254:821 (1991); deVos, et al., Science 255:306 (1992); Fuh, et al., Science 256:1677 (1992)). Accordingly, antagonist variants of hVEGF are selected in which one receptor binding site of hVEGF (typically the site involved in the initial binding of hVEGF to hVEGFr) remains unmodified (or if modified is varied to enhance binding), while a second receptor binding site of hVEGF typically is modified by nonconservative amino acid residue substitution(s) or deletion(s) in order to render that binding site inoperative.

Receptor binding domains in hVEGF and hVEGF binding domains in hVEGFr are determined by methods known in the art, including X-ray studies, mutational analyses, and antibody binding studies. The mutational approaches include the techniques of random saturation mutagenesis coupled with selection of escape mutants, and insertional mutagenesis. Another strategy suitable for identifying receptor-binding domains in ligands is known as alanine (Ala)-scanning mutagenesis. Cunningham, et al., Science 244, 1081-1985 (1989). This method involves the identification of regions that contain charged amino acid side chains. The charged residues in each region identified (i.e. Arg, Asp, His, Lys, and Glu) are replaced (one region per mutant molecule) with Ala and the receptor binding of the obtained ligands is tested, to assess the importance of the particular region in receptor binding. A further powerful method for the localization of receptor binding domains is through the use of neutralizing anti-hVEGF antibodies. Kim, et al., Growth Factors 7:53 (1992). Usually a combination of these and similar methods is used for localizing the domains involved in receptor binding.

The term "amino acid sequence variant" used in reference to hVEGF refers to polypeptides having amino acid sequences that differ to some extent from the amino acid sequences of the native forms of hVEGF. Ordinarily, antagonist amino acid sequence variants will possess at least about 70% homology with at least one receptor binding domain of a native hVEGF, and preferably, they will be at least about 80%, more preferably at least about 90% homologous with a receptor binding domain of a nativ hVEGF. Th amino acid

sequence variants possess substitutions, deletions, and/or insertions at certain positions within th amino acid sequence of native hVEGF, such that the variants retain the ability to bind to hVEGF receptor (and thus compete with native hVEGF for binding to hVEGF receptor) but fail to induce one or more of the biological effects of hVEGF, such as endothelial cell proliferation, angiogenesis, or vascular permeability.

"Homology" is defined as the percentage of residues in the amino acid sequence variant that are identical with the residues in the amino acid sequence of a receptor binding domain of a native hVEGF after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent homology. Methods and computer programs for the alignment are well known in the art. One such computer program is "Align 2", authorec by Genentech, Inc., which was filed with user documentation in the United States Copyright Office, Washington, DC 20559, on December 10, 1991. Substitutional variants are those that have at least one amino acid residue in a native sequence removed and a different amino acid inserted in its place at the same position. The substitutions may be single, where only one amino acid in the molecule has been substituted, or they may be multiple, where two or more amino acids have been substituted in the same molecule.

Insertional variants are those with one or more amino acids inserted immediately adjacent to an amino acid at a particular position in a native sequence. Immediately adjacent to an amino acid means connected to either the α-carboxy or α-amino functional group of the amino acid.

Deletional variants are those with one or more amino acid residues in a native sequence removed. Ordinarily, deletional variants will have one or two amino acid residues deleted in a particular region of the molecule.

Fragments and amino acid sequence variants of hVEGF are readily prepared by methods known in the art, such as by site directed mutagenesis of the DNA encoding the native factor. The mutated DNA is inserted into an appropriate expression vector, and host cells are then transfected with the recombinant vector. The recombinant host cells and grown in suitable culture medium, and the desired fragment or amino acid sequence variant expressed in the host cells then is recovered from the recombinant cell culture by chromatographic or other purification methods.

Alternatively, fragments and amino acid variants of hVEGF are prepared in vitro, for example by proteolysis of native hVEGF, or by synthesis using standard solid-phase peptide synthesis procedures as described by Merrifield (J. Am. Chem. Soc. 85:2149 [1963]), although other equivalent chemical syntheses known in the art may be used. Solid-phase synthesis is initiated from the C-terminus of the peptide by coupling a protected α-amino acid to a suitable resin. The amino acids are coupled to the peptide chain using techniques well known in the art for the formation of peptide bonds.

Therapeutic Uses

For therapeutic applications, the antagonists of the invention are administered to a mammal, preferably a human, in a pharmaceutically acceptabl dosage form, including those that may be administered to a human intravenously as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intra-cerobrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes. The antagonists also are suitably administered by intratumoral, peritumoral, intralesional, or perilesional routes, to exert local as well as systemic therapeutic effects. The intraperitoneal route is expected to be particularly useful, for example, in the treatment of ovarian tumors.

Such dosage forms encompass pharmaceutically acceptable carriers that are inherently nontoxic and nontherapeutic. Examples of such carriers include ion exchangers, alumina, aluminum stearate, lecithin, serum proteins, such as human serum albumin, buffer substances such as phosphates, glycine, sorbic acid, potassium sorbate, partial glyceride mixtures of saturated vegetable fatty acids, water, salts, or electrolytes such as protamine sulfate, disodium hydrogen phosphate, potassium hydrogen phosphate, sodium chloride, zinc salts, colloidal silica, magnesium trisilicate, polyvinyl pyrrolidone, cellulose-based substances, and polyethylene glycol. Carriers for topical or gel-based forms of antagonist include polysaccharides such as sodium carboxymethylcellulose or methylcellulose, polyvinylpyrrolidone, polyacrylates, polyoxyethylene-polyoxypropylene-block polymers, polyethylene glycol, and wood wax alcohols. For all administrations, conventional depot forms are suitably used. Such forms include, for example, microcapsules, nano-capsules, liposomes, plasters, inhalation forms, nose sprays, sublingual tablets, and sustained-release preparations. The antagonist will typically be formulated in such vehicles at a concentration of about 0.1 mg/ml to 100 mg/ml.

Suitable examples of sustained release preparations include semipermeable matrices of solid hydrophobic polymers containing the antagonist, which matrices are in the form of shaped articles, e.g. films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate) as described by Langer et al., J. Biomed. Mater. Res. 15:167 (1981) and Langer, Chem. Tech., 12: 98-105 (1982), or poly(vinylalcohol), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and gamma ethyl-L-glutamate (Sidman et al., Biopolymers, 22:547 (1983), non-degradable ethylene-vinyl acetate (Langer et al., supra), degradable lactic acid-glycolic acid copolymers such as the Lupron Depot™ (injectable micropheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated polypeptide antagonists remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37°C, resulting in a loss of biological activity and

possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on th mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S-S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

Sustained-release hVEGF antagonist compositions also include liposomally entrapped antagonist antibodies and hVEGFr. Liposomes containing the antagonists are prepared by methods known in the art, such as described in Epstein, et al., Proc. Natl. Acad. Sci. USA, 82:3688 (1985); Hwang, et al., Proc. Natl. Acad. Sci. USA, 77:4030 (1980); U.S. Patent No. 4,485,045; U.S. Patent No. 4,544,545. Ordinarily the liposomes are the small (about 200-800 Angstroms) unilamelar type in which the lipid content is greater than about 30 mol.% cholesterol, the selected proportion being adjusted for the optimal HRG therapy. Liposomes with enhanced circulation time are disclosed in U.S. Patent No. 5,013,556.

Another use of the present invention comprises incorporating an hVEGF antagonist into formed articles. Such articles can be used in modulating endothelial cell growth and angiogenesis. In addition, tumor invasion and metastasis may be modulated with these articles.

For the prevention or treatment of disease, the appropriate dosage of antagonist will depend on the type of disease to be treated, as defined above, the severity and course of the disease, whether the antibodies are administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the antagonist, and the discretion of the attending physician. The antagonist is suitably administered to the patient at one time or over a series of treatments.

The hVEGF antagonists are useful in the treatment of various neoplastic and non-neoplastic diseases and disorders. Neoplasms and related conditions that are amenable to treatment include breast carcinomas, lung carcinomas, gastric carcinomas, esophageal carcinomas, colorectal carcinomas, liver carcinomas, ovarian carcinomas, thecomas, arrhenoblastomas, cervical carcinomas, endometrial carcinoma, endometrial hyperplasia, endometriosis, fibrosarcomas, choriocarcinoma, head and neck cancer, nasopharyngeal carcinoma, laryngeal carcinomas, hepatoblastoma, Kaposi's sarcoma, melanoma, skin carcinomas, hemangioma, cavernous hemangioma, hemangioblastoma, pancreas carcinomas, retinoblastoma, astrocytoma, glioblastoma, Schwannoma, oligodendroglioma, medulloblastoma, neuroblastomas, rhabdomyosarcoma, osteogenic sarcoma, leiomyosarcomas, urinary tract carcinomas, thyroid carcinomas, Wilm's tumor, renal cell carcinoma, prostate carcinoma, abnormal vascular proliferation associated with phakomatoses, edema (such as that associated with brain tumors), and Meigs' syndrome.

Non-neoplastic conditions that ar amenable to treatment include rheumatoid arthritis, psoriasis, atherosclerosis, diabetic and ther retinopathies, retrolental fibroplasia, n ovascular glaucoma, thyroid hyperplasias (including Grave's disease), corneal and other tissue transplantation, chronic inflammation, lung inflammation, n phrotic syndrome, pr eclampsia, ascites, pericardial effusion (such as that associated with pericarditis), and pleural effusion.

Depending on the type and severity of the disease, about 1 μg/kg to 15 mg/kg of antagonist is an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. A typical daily dosage might range from about 1 μg/kg to 100 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment is repeated until a desired suppression of disease symptoms occurs. However, other dosage regimens may be useful. The progress of this therapy is easily monitored by conventional techniques and assays, including, for example, radiographic tumor imaging.

According to another embodiment of the invention, the effectiveness of the antagonist in preventing or treating disease may be improved by administering the antagonist serially or in combination with another agent that is effective for those purposes, such as tumor necrosis factor (TNF), an antibody capable of inhibiting or neutralizing the angiogenic activity of acidic or basic fibroblast growth factor (FGF) or hepatocyte growth factor (HGF), an antibody capable of inhibiting or neutralizing the coagulant activities of tissue factor, protein C, or protein S (see Esmon, et al., PCT Patent Publication No. WO 91/01753, published 21 February 1991), or one or more conventional therapeutic agents such as, for example, alkylating agents, folic acid antagonists, anti-metabolites of nucleic acid metabolism, antibiotics, pyrimidine analogs, 5-fluorouracil, purine nucleosides, amines, amino acids, triazol nucleosides, or corticosteroids. Such other agents may be present in the composition being administered or may be administered separately. Also, the antagonist is suitably administered serially or in combination with radiological treatments, whether involving irradiation or administration of radioactive substances.

In one embodiment, vascularization of tumors is attacked in combination therapy. One or more hVEGF antagonists are administered to tumor-bearing patients at therapeutically effective doses as determined for example by observing necrosis of the tumor or its metastatic foci, if any. This therapy is continued until such time as no further beneficial effect is observed or clinical examination shows no trace of the tumor or any metastatic foci. Then TNF is administered, alone or in combination with an auxiliary agent such as alpha-, beta-, or gamma-interferon, anti-HER2 antibody, heregulin, anti-heregulin antibody, D-factor, interleukin-1 (IL-1), interleukin-2 (IL-2), granulocyte-macrophage colony stimulating factor (GM-CSF), or agents that promote microvascular coagulation in tumors, such as anti-protein

C antibody, anti-protein S antibody, or C4b binding protein (see Esmon, et al., PCT Patent Publication No. WO 91/01753, published 21 February 1991), or heat or radiation.

Since th auxiliary agents will vary in their effectiveness it is desireable to compare their impact on the tumor by matrix screening in conventional fashion. The administration of hVEGF antagonist and TNF is repeated until the desired clinical effect is achieved. Alternatively, the hVEGF antagonist(s) are administered together with TNF and, optionally, auxiliary agent(s). In instances where solid tumors are found in the limbs or in other locations susceptible to isolation from the general circulation, the therapeutic agents described herein are administered to the isolated tumor or organ. In other embodiments, a FGF or platelet-derived growth factor (PDGF) antagonist, such as an anti-FGF or an anti-PDGF neutralizing antibody, is administered to the patient in conjunction with the hVEGF antagonist. Treatment with hVEGF antagonists optimally may be suspended during periods of wound healing or desirable neovascularization.

Other Uses

The anti-hVEGF antibodies of the invention also are useful as affinity purification agents. In this process, the antibodies against hVEGF are immobilized on a suitable support, such a Sephadex resin or filter paper, using methods well known in the art. The immobilized antibody then is contacted with a sample containing the hVEGF to be purified, and thereafter the support is washed with a suitable solvent that will remove substantially all the material in the sample except the hVEGF, which is bound to the immobilized antibody. Finally, the support is washed with another suitable solvent, such as glycine buffer, pH 5.0, that will release the hVEGF from the antibody.

The following examples are offered by way of illustration only and are not intended to limit the invention in any manner.

EXAMPLE 1

Preparation of Anti- hVEGF Monoclonal Antibodies

To obtain hVEGF conjugated to keyhole limpet hemocyanin (KLH) for immunization, recombinant hVEGF (165 amino acids), Leung, et al., Science 246:1306 (1989), was mixed with KLH at a 4:1 ratio in the presence of 0.05% glutaraldehyde and the mixture was incubated at room temperature for 3 hours with gentle stirring. The mixture then was dialyzed against phosphate buffered saline (PBS) at 4° C. overnight.

Balb/c mice were immunized four times every two weeks by intraperitoneal injections with 5 μg of hVEGF conjugated to 20 μg of KLH, and were boosted with the same dose of hVEGF conjugated to KLH four days prior to cell fusion.

Spleen cells from the immunized mice were fused with P3X63Ag8U.1 myeloma cells, Yelton, et al., Curr. Top. Microbiol. Immunol. 81:1 (1978), using 35% polyethylene glycol (PEG) as described. Yarmush, et al., Proc. Nat. Acad. Sci. 77:2899 (1980). Hybridomas were selected in HAT medium.

Supernatants from hybridoma cell cultures were screened for anti-hVEGF antibody production by an ELISA assay using hVEGF-coated microtiter plates. Antibody that was bound to hVEGF in each of the wells was determined using alkaline phosphatase-conjugated goat anti-mouse IgG immunoglobulin and the chromogenic substrate p-nitrophenyl phosphate. Harlow & Lane, Antibodies: A Laboratory Manual, p.597 (Cold Spring Harbor Laboratory, 1988). Hybridoma cells thus determined to produce anti-hVEGF antibodies were subcloned by limiting dilution, and two of those clones, designated A4.6.1 and B2.6.2, were chosen for further studies.

EXAMPLE 2

Characterization of Anti-hVEGF Monoclonal Antibodies

A. Antigen Specificity

The binding specificities of the anti-hVEGF monoclonal antibodies produced by the A4.6.1 and B2.6.2 hybridomas were determined by ELISA. The monoclonal antibodies were added to the wells of microtiter plates that previously had been coated with hVEGF, FGF, HGF, or epidermal growth factor (EGF). Bound antibody was detected with peroxidase conjugated goat anti-mouse IgG immunoglobulins. The results of those assays confirmed that the monoclonal antibodies produced by the A4.6.1 and B2.6.2 hybridomas bind to hVEGF, but not detectably to those other protein growth factors.

B. Epitope Mapping

A competitive binding ELISA was used to determine whether the monoclonal antibodies produced by the A4.6.1 and B2.6.2 hybridomas bind to the same or different epitopes (sites) within hVEGF. Kim, et al., Infect. Immun. 57:944 (1989). Individual unlabeled anti-hVEGF monoclonal antibodies (A4.6.1 or B2.6.2) or irrelevant anti-HGF antibody (IgG1 isotype) were added to the wells of microtiter plates that previously had been coated with hVEGF. Biotinylated anti-hVEGF monoclonal antibodies (BIO-A4.6.1 or BIO-B2.6.2) were then added. The ratio of biotinylated antibody to unlabeled antibody was 1:1000. Binding of the biotinylated antibodies was visualized by the addition of avidin-conjugated peroxidase, followed by o-phenylenediamine dihydrochloride and hydrogen peroxide. The color reaction, indicating the amount of biotinylated antibody bound, was determined by measuring the optical density (O.D) at 495 nm wavelength.

As shown in Figure 1, in each case, the binding of the biotinylated anti-hVEGF antibody was inhibited by the corresponding unlabeled antibody, but not by the other unlabeled anti-hVEGF antibody or the anti-HGF antibody. These results indicate that the monoclonal antibodies produced by the A4.6.1 and B2.6.2 hybridomas bind to different epitopes within hVEGF.

C. Isotyping

The isotypes of the anti-hVEGF monoclonal antibodies produced by the A4.6.1 and B2.6.2 hybridomas were determined by ELISA. Samples of culture medium (supernatant) in

which each of the hybridomas was growing were added to the wells of microtit r plates that had previously been coated with hVEGF. The captured anti-hVEGF monoclonal antibodies were incubated with different isotype-specific alkaline phosphatase-conjugated goat anti-mouse immunoglobulins, and the binding of the conjugated antibodies to the anti-hVEGF monoclonal antibodies was determined by the addition of p-nitrophenyl phosphate. The color reaction was measured at 405 nm with an ELISA plate reader.

By that method, the isotype of the monoclonal antibodies produced by both the A4.6.1 and B2.6.2 hybridomas was determined to be IgG1.

D. Binding Affinity

The affinities of the anti-hVEGF monoclonal antibodies produced by the A4.6.1 and B2.6.2 hybridomas for hVEGF were determined by a competitive binding assays. A predetermined sub-optimal concentration of monoclonal antibody was added to samples containing 20,000 - 40,000 cpm ^{125}I-hVEGF (1 - 2 ng) and various known amounts of unlabeled hVEGF (1 - 1000 ng). After 1 hour at room temperature, 100 μl of goat anti-mouse Ig antisera (Pel-Freez, Rogers, AR USA) were added, and the mixtures were incubated another hour at room temperature. Complexes of antibody and bound protein (immune complexes) were precipitated by the addition of 500 μl of 6% polyethylene glycol (PEG, mol. wt. 8000) at 4° C., followed by centrifugation at 2000 x G. for 20 min. at 4° C. The amount of ^{125}I-hVEGF bound to the anti-hVEGF monoclonal antibody in each sample was determined by counting the pelleted material in a gamma counter.

Affinity constants were calculated from the data by Scatchard analysis. The affinity of the anti-hVEGF monoclonal antibody produced by the A4.6.1 hybridoma was calculated to be 1.2 x 10^9 liters/mole. The affinity of the anti-hVEGF monoclonal antibody produced by the B2.6.2 hybridoma was calculated to be 2.5 x 10^9 liters/mole.

E. Inhibition of hVEGF Mitogenic Activity

Bovine adrenal cortex capillary endothelial (ACE) cells, Ferrara, et al., Proc. Nat. Acad. Sci. 84:5773 (1987), were seeded at a density of 10^4 cells/ml in 12 multiwell plates, and 2.5 ng/ml hVEGF was added to each well in the presence or absence of various concentrations of the anti-hVEGF monoclonal antibodies produced by the A4.6.1 or B2.6.2 hybridomas, or an irrelevant anti-HGF monoclonal antibody. After culturing 5 days, the cells in each well were counted in a Coulter counter. As a control, ACE cells were cultured in the absence of added hVEGF.

As shown in Figure 2, both of the anti-hVEGF monoclonal antibodies inhibited the ability of the added hVEGF to support the growth or survival of the bovine ACE cells. The monoclonal antibody produced by the A4.6.1 hybridoma completely inhibited the mitogenic activity of hVEGF (greater than about 90% inhibition), whereas the monoclonal antibody produced by the B2.6.2 hybridoma only partially inhibited the mitogenic activity of hVEGF.

F. Inhibition of hVEGF Binding

Bovine ACE cells were seeded at a density of 2.5 x 10^4 cells/0.5 ml/well in 24 well microtiter plates in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% calf serum, 2 mM glutamin , and 1 ng/ml basic fibroblast growth factor. After culturing overnight, the cells were washed once in binding buffer (equal volumes of DMEM and F12 medium plus 25 mM HEPES and 1% bovine serum albumin) at 4° C.

12,000 cpm ^{125}I-hVEGF (approx. 5 x 10^4 cpm/ng/ml) was preincubated for 30 minutes with 5 μg of the anti-hVEGF monoclonal antibody produced by the A4.6.1, B2.6.2, or A2.6.1 hybridoma (250 μl total volume), and thereafter the mixtures were added to the bovine ACE cells in the microtiter plates. After incubating the cells for 3 hours at 4° C., the cells were washed 3 times with binding buffer at 4° C., solubilized by the addition of 0.5 ml 0.2 N. NaOH, and counted in a gamma counter.

As shown in Figure 3 (upper), the anti-hVEGF monoclonal antibodies produced by the A4.6.1 and B2.6.2 hybridomas inhibited the binding of hVEGF to the bovine ACE cells. In contrast, the anti-hVEGF monoclonal antibody produced by the A2.6.1 hybridoma had no apparent effect on the binding of hVEGF to the bovine ACE cells. Consistent with the results obtained in the cell proliferation assay described above, the monoclonal antibody produced by the A4.6.1 hybridoma inhibited the binding of hVEGF to a greater extent than the monoclonal antibody produced by the B2.6.2 hybridoma.

As shown in Figure 3 (lower), the monoclonal antibody produced by the A4.6.1 hybridoma completely inhibited the binding of hVEGF to the bovine ACE cells at a 1:250 molar ratio of hVEGF to antibody.

G. Cross-reactivity with other VEGF isoforms

To determine whether the anti-hVEGF monoclonal antibody produced by the A4.6.1 hybridoma is reactive with the 121- and 189-amino acid forms of hVEGF, the antibody was assayed for its ability to immunoprecipate those polypeptides.

Human 293 cells were transfected with vectors comprising the nucleotide coding sequence of the 121- and 189-amino acid hVEGF polypeptides, as described. Leung, et al., Science 246:1306 (1989). Two days after transfection, the cells were transferred to medium lacking cysteine and methionine. The cells were incubated 30 minutes in that medium, then 100 μCi/ml of each ^{35}S-methionine and ^{35}S-cysteine were added to the medium, and the cells were incubated another two hours. The labeling was chased by transferring the cells to serum free medium and incubating three hours. The cell culture media were collected, and the cells were lysed by incubating for 30 minutes in lysis buffer (150 mM NaCl, 1% NP40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris, pH 8.0). Cell debris was removed from the lysates by centrifugation at 200 x G. for 30 minutes.

500 μl samples of cell culture media and cell lysates were incubated with 2 μl of A4.6.1 hybridoma antibody (2.4 mg/ml) for 1 hour at 4° C., and then were incubated with

5 μl of rabbit anti-mouse IgG immunoglobulin for 1 hour at 4° C. Immune complexes of ^{35}S-labeled hVEGF and anti-hVEGF monoclonal antibody were precipitated with protein-A Sepharose (Pharmacia), then subjected to SDS - 12% polyacrylamide gel lectrophoresis under reducing conditions. Th gel was exposed to x-ray film for analysis of the immunoprecipitated, radiolabeled proteins by autoradiography.

The results of that analysis indicated that the anti-hVEGF monoclonal antibody produced by the A4.6.1 hybridoma was cross-reactive with both the 121- and 189-amino acid forms of hVEGF.

EXAMPLE 3

Preparation of hVEGF Receptor - IgG Fusion Protein

The nucleotide and amino acid coding sequences of the flt hVEGF receptor are disclosed in Shibuya, et al., Oncogene 5:519-524 (1990). The coding sequence of the extracellular domain of the flt hVEGF receptor was fused to the coding sequence of human IgG1 heavy chain in a two-step process.

Site-directed mutagenesis was used to introduce a BstBI restriction into DNA encoding flt at a site 5' to the codon for amino acid 759 of flt, and to convert the unique BstEII restriction site in plasmid pBSSK⁻FC, Bennett, et al., J. Biol. Chem. 266:23060-23067 (1991), to a BstBI site. The modified plasmid was digested with EcoRI and BstBI and the resulting large fragment of plasmid DNA was ligated together with an EcoRI-BstBI fragment of the flt DNA encoding the extracellular domain (amino acids 1-758) of the flt hVEGF receptor.

The resulting construct was digested with ClaI and NotI to generate an approximately 3.3 kb fragment, which is then inserted into the multiple cloning site of the mammalian expression vector pHEBO2 (Leung, et al., Neuron 8:1045 (1992) by ligation. The ends of 3.3. kb fragment are modified, for example by the addition of linkers, to obtain insertion of the fragment into the vector in the correct orientation for expression.

Mammalian host cells (for example, CEN4 cells (Leung, et al. supra) are transfected with the pHEBO2 plasmid containing the flt insert by electroporation. Transfected cells are cultured in medium containing about 10% fetal bovine serum, 2 mM glutamine, and antibiotics, and at about 75% confluency are transferred to serum free medium. Medium is conditioned for 3-4 days prior to collection, and the flt-IgG fusion protein is purified from the conditioned medium by chromatography on a protein-A affinity matrix essentially as described in Bennett, et al., J. Biol. Chem. 266:23060-23067 (1991).

EXAMPLE 4

Inhibition of Tumor Growth with hVEGF Antagonists

Various human tumor cell lines growing in culture were assayed for production of hVEGF by ELISA. Ovary, lung, colon, gastric, breast, and brain tumor cell lines were found

to produce hVEGF. Three cell lines that produced hVEGF, NEG 55 (also referred to as G55) (human glioma cell line obtained from Dr. M. Westphal, Department of Neurosurgery, University Hospital Eppendor, Hamburg, Germany, also referred to as G55), A-673 (human rhabdomyosarcoma cell line obtained from the American Type Culture Collection (ATCC), Rockville, Maryland USA 20852 as cell line number CRL 1598), and SK-LMS-1 (leiomyosarcoma cell line obtained from the ATCC as cell line number HTB 88), were used for further studies.

Six to ten week old female Beige/nude mice (Charles River Laboratory, Wilmington, Massachusetts USA) were injected subcutaneously with 1 - 5 x 10^6 tumor cells in 100-200 μl PBS. At various times after tumor growth was established, mice were injected intraperitoneally once or twice per week with various doses of A4.6.1 anti-hVEGF monoclonal antibody, an irrelevant anti-gp120 monoclonal antibody (5B6), or PBS. Tumor size was measured every week, and at the conclusion of the study the tumors were excised and weighed.

The effect of various amounts of A4.6.1 anti-hVEGF monoclonal antibody on the growth of NEG 55 tumors in mice is shown in Figures 4 and 5. Figure 4 shows that mice treated with 25 μg or 100 μg of A4.6.1 anti-hVEGF monoclonal antibody beginning one week after inoculation of NEG 55 cells had a substantially reduced rate of tumor growth as compared to mice treated with either irrelevant antibody or PBS. Figure 5 shows that five weeks after inoculation of the NEG 55 cells, the size of the tumors in mice treated with A4.6.1 anti-hVEGF antibody was about 50% (in the case of mice treated with 25 μg dosages of the antibody) to 85% (in the case of mice treated with 100 μg dosages of the antibody) less than the size of tumors in mice treated with irrelevant antibody or PBS.

The effect of A4.6.1 anti-hVEGF monoclonal antibody treatment on the growth of SK-LMS-1 tumors in mice is shown in Figure 6. Five weeks after innoculation of the SK-LMS-1 cells, the average size of tumors in mice treated with the A4.6.1 anti-hVEGF antibody was about 75% less than the size tumors in mice treated with irrelevant antibody or PBS.

The effect of A4.6.1 anti-hVEGF monoclonal antibody treatment on the growth of A673 tumors in mice is shown in Figure 7. Four weeks after innoculation of the A673 cells, the average size of tumors in mice treated with A4.6.1 anti-hVEGF antibody was about 60% (in the case of mice treated with 10 μg dosages of the antibody) to greater than 90% (in the case of mice treated with 50-400 μg dosages of the antibody) less than the size of tumors in mice treated with irrelevant antibody or PBS.

EXAMPLE 5

Analysis of the Direct Effect of Anti-hVEGF Antibody on Tumor Cells Growing in Culture

NEG55 human glioblastoma cells or A673 rhabdomyosarcoma cells were seeded at a density of 7 x 10^3 cells/well in multiwells plates (12 wells/plate) in F12/DMEM medium

containing 10% fetal calf serum, 2mM glutamine, and antibi tics. A4.6.1 anti-hVEGF antibody then was added to the cell cultures to a final concentration of 0 - 20.0 μg antibody/ml. After five days, the cells growing in the wells were dissociated by exposure to trypsin and counted in a Coulter counter.

Figures 8 and 9 show the results of those studies. As is apparent, the A4.6.1 anti-hVEGF antibody did not have any significant effect on the growth of the NEG55 or A673 cells in culture. These results indicate that the A4.6.1 anti-hVEGF antibody is not cytotoxic, and strongly suggest that the observed anti-tumor effects of the antibody are due to its inhibition of VEGF-mediated neovascularization.

EXAMPLE 6

Effect of Anti-hVEGF Antibody on
Endothelial Cell Chemotaxis

Chemotaxis of endothelial cells and others cells, including monocytes and lymphocytes, play an important role in the pathogenesis of rheumatoid arthritis. Endothelial cell migration and proliferation accompany the angiogenesis that occurs in the rheumatoid synovium. Vascularized tissue (pannus) invades and destroys the articular cartilage.

To determine whether hVEGF antagonists interfere with this process, we assayed the effect of the A4.6.1 anti-hVEGF antibody on endothelial cell chemotaxis stimulated by synovial fluid from patients having rheumatoid arthritis. As a control, we also assayed the effect of the A4.6.1 anti-hVEGF antibody on endothelial cell chemotaxis stimulated by synovial fluid from patients having osteoarthritis (the angiogenesis that occurs in rheumatoid arthritis does not occur in osteoarthritis).

Endothelial cell chemotaxis was assayed using modified Boyden chambers according to established procedures. Thompson, et al., Cancer Res. 51:2670 (1991); Phillips, et al., Proc. Exp. Biol. Med. 197:458 (1991). About 10^4 human umbilical vein endothelial cells were allowed to adhere to gelatin-coated filters (0.8 micron pore size) in 48-well multiwell microchambers in culture medium containing 0.1% fetal bovine serum. After about two hours, the chambers were inverted and test samples (rheumatoid arthritis synovial fluid, osteoarthritis synovial fluid, basic FGF (bFGF) (to a final concentration of 1 μg/ml), or PBS) and A4.6.1 anti-hVEGF antibody (to a final concentration of 10 μg/ml) were added to the wells. After two to four hours, cells that had migrated were stained and counted.

Figure 10 shows the averaged results of those studies. The values shown in the column labeled "Syn. Fluid" and shown at the bottom of the page for the controls are the average number of endothelial cells that migrated in the presence of synovial fluid, bFGF, or PBS alone. The values in the column labeled "Syn. Fluid + mAB VEGF" are the average number of endothelial cells that migrated in the presence of synovial fluid plus added A4.6.1 anti-hVEGF antibody. The values in the column labeled "% Suppression" indicate the percentage reduction in synovial fluid-induced endothelial cell migration resulting from the

addition of anti-hVEGF antibody. As indicated, the anti-hVEGF antibody significantly inhibited the ability of rheumatoid arthritis synovial fluid (53.40 average percentage inhibition), but not osteorthritis synovial fluid (13.64 average percentage inhibition), to induce endothelial cell migration.

What is claimed is:

1. A composition comprising a hVEGF antagonist, provided however that th antagonist is not the flt or flk-1 r KDR receptor or a neutralizing anti-hVEGF antibody.

2. A composition of claim 1 including a polypeptide comprising an antibody amino acid sequence that is capable of binding to a hVEGF receptor and that competes with hVEGF for binding to the receptor.

3. A composition of claim 1 including a polypeptide comprising an antibody amino acid sequence that is capable of binding to hVEGF and that interferes with the binding of hVEGF to a hVEGF receptor.

4. A monoclonal antibody amino acid sequence capable of specifically binding to a hVEGFr or a hVEGF-hVEGFr complex.

5. A monoclonal antibody amino acid sequence of claim 4 which inhibits the mitogenic activity of a hVEGF or inhibits the binding of a hVEGF to bovine ACE cells.

6. A monoclonal antibody amino acid sequence of claim 5 which inhibits the mitogenic activity of a hVEGF at least about 90%.

7. A monoclonal antibody amino acid sequence of claim 4 which is capable of binding to hVEGFr.

8. A monoclonal antibody amino acid sequence of claim 7 which is monovalent for binding to hVEGFr.

9. A monoclonal antibody amino acid sequence of claim 4 which is heterospecific.

10. A monoclonal antibody sequence of claim 9 which is capable of binding to an antigen other than hVEGF, hVEGFr, and hVEGF-hVEGFr complex.

11. A monoclonal antibody amino acid sequence of claim 4 which comprises an amino acid sequence from the Fc domain of either the IgA, IgD, IgE, IgG1, IgG2, IgG3, IgG4 or IgM heavy chains.

12. A monoclonal antibody amino acid sequence of claim 4 which comprises a human Fc domain.

13. A monoclonal antibody amino acid sequence of claim 12 which further comprises a murine Fv domain capable of binding hVEGF, hVEGFr, or hVEGF-hVEGFr complex.

14. A monoclonal antibody amino acid sequence of claim 4 further comprising a non-immunoglobulin polymer.

15. A monoclonal antibody amino acid sequence of claim 4 further comprising a cytotoxic moiety or an amino acid sequence of a cytokine.

16. A monoclonal antibody amino acid sequence of claim 15 wherein the cytotoxic moiety or the amino acid sequence of the cytokine is substituted for an Fc sequence.

17. A monoclonal antibody amino acid sequence of claim 15 having a cytotoxic moiety that is a polypeptide toxin.

18. A monoclonal antibody amino acid sequence of claim 15 having a cytotoxic moiety that is capable of Fc eff ctor function or of recruiting an immune cell.

19. A monoclonal antibody amino acid sequence of claim 18 wherein the cytoxic moiety is a polypeptide capable of binding complement.

20. A monoclonal antibody amino acid sequence of claim 18 wherein the cytotoxic moiety is a polypeptide capable of binding CD3, CD18, CD11a, CDIIb, or CD11c.

21. A monoclonal antibody amino acid sequence of claim 4 which is capable of binding to hVEGF-hVEGFr complex but not to hVEGF or to hVEGFr alone.

22. A monoclonal antibody amino acid sequence of claim 21 further comprising a cytotoxic moiety.

23. A monoclonal antibody amino acid sequence of claim 4 which is capable of binding to hVEGFr and which antagonizes the effect of hVEGF on the hVEGFr.

24. A monoclonal antibody amino acid sequence of claim 4 further comprising a physiologically acceptable vehicle and which is sterile, present in a substantially isotonic solution, and stored in a container hermetically sealed with an elastomeric stopper.

25. A monoclonal antibody sequence of claim 24 in a kit together with a written insert containing instructions for therapeutic use.

26. A polypeptide comprising an amino acid sequence encoding a hVEGFr and an immunoglobulin chain.

27. A method of treatment of a tumor in a mammal comprising administering to the mammal a therapeutically effective amount of a hVEGF antagonist sufficient to reduce the size of the tumor.

28. A method of claim 27 wherein the hVEGF antagonist is an anti-hVEGFr antibody.

29. A method of claim 27 wherein the hVEGF antagonist is an anti-hVEGF-hVEGFr complex antibody.

30. A method of claim 27 wherein the hVEGF antagonist comprises an amino acid sequence encoding the extracellular domain of a hVEGFr.

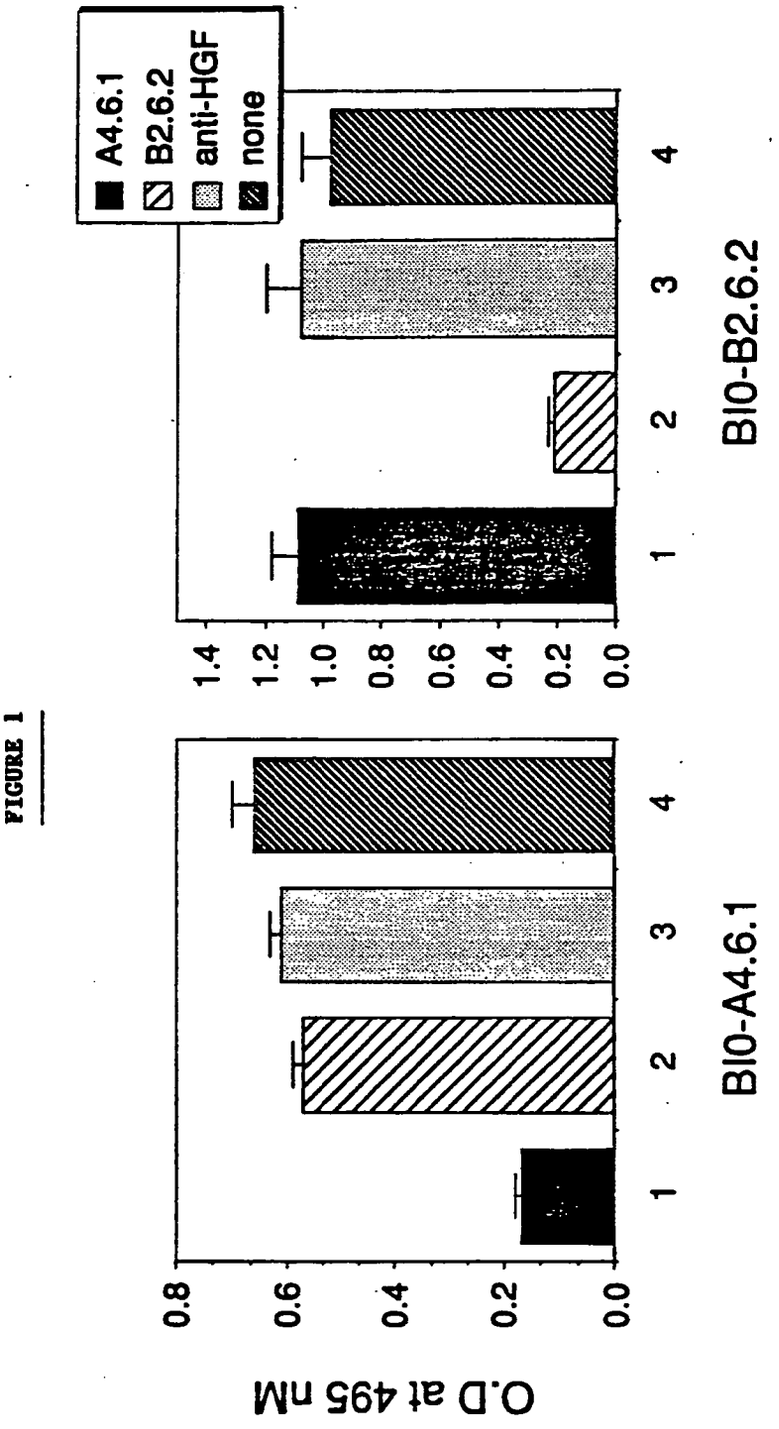


FIGURE 1

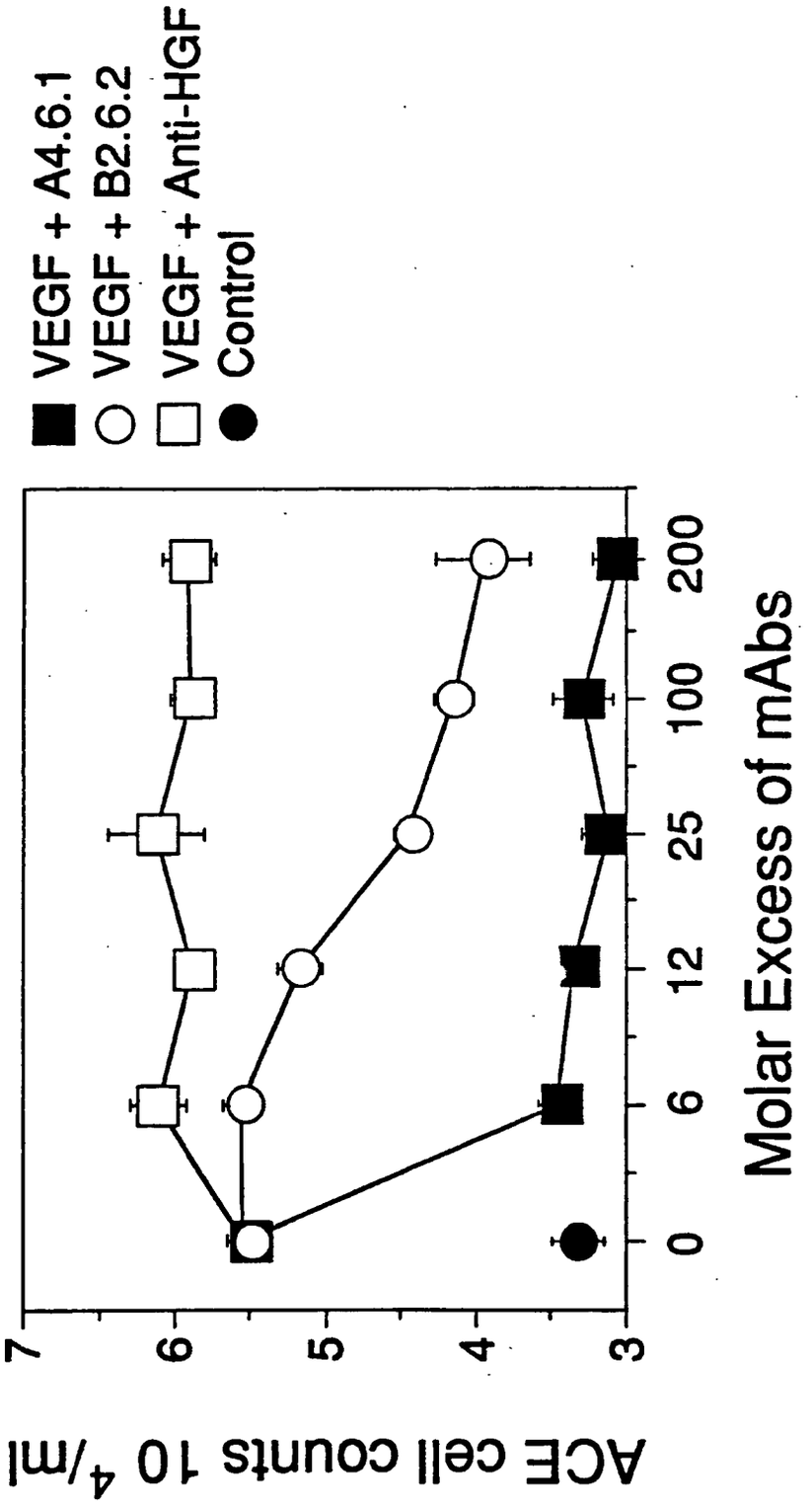


FIGURE 2

FIGURE 3

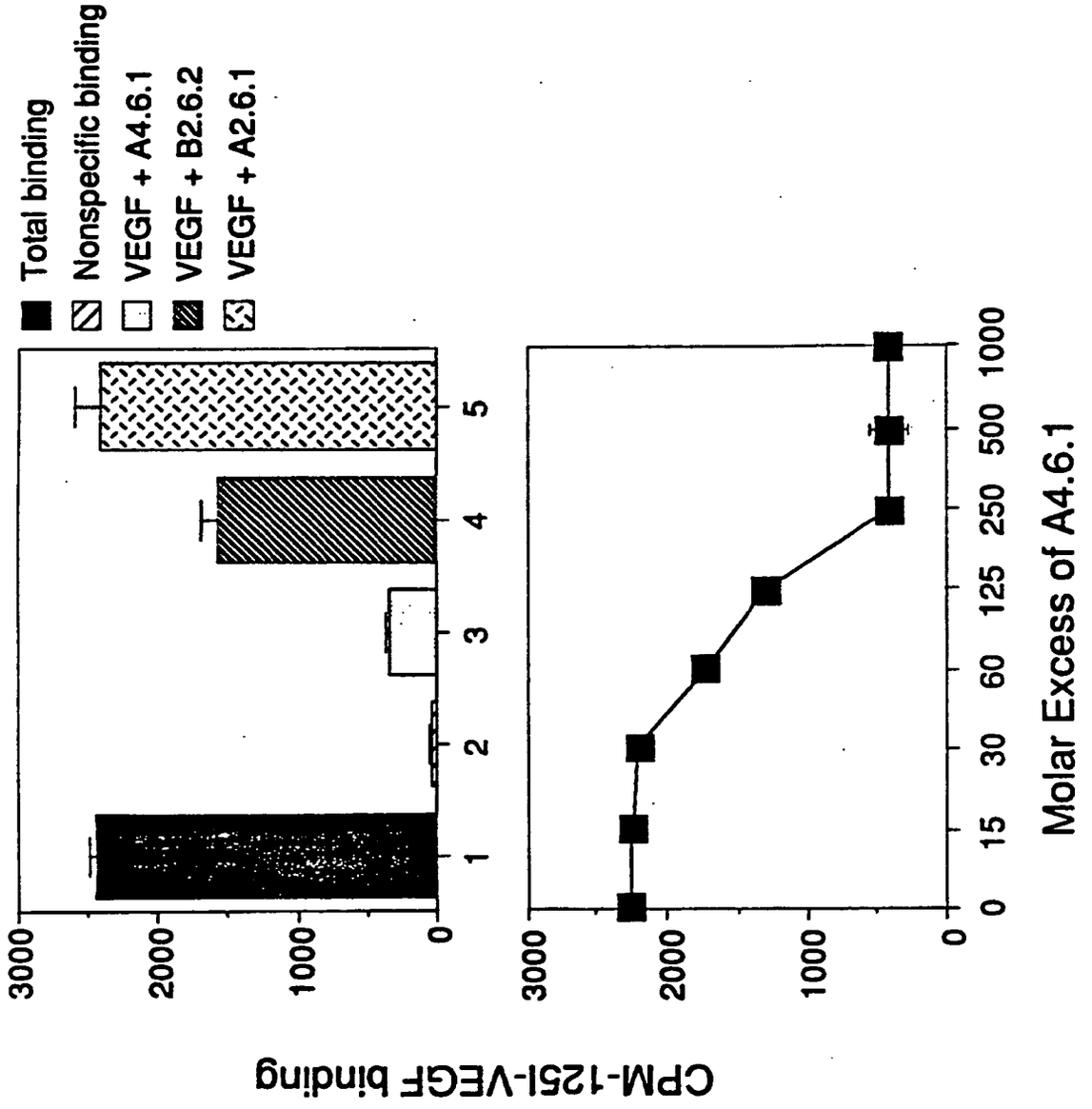


Figure 4

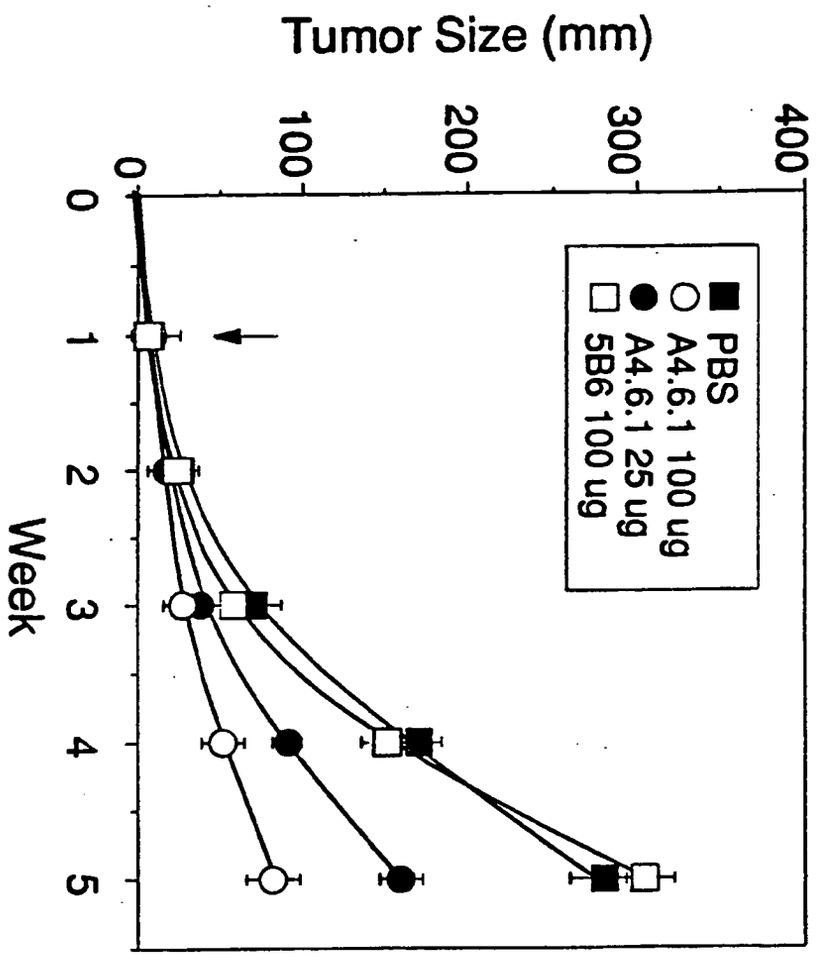


Figure 5

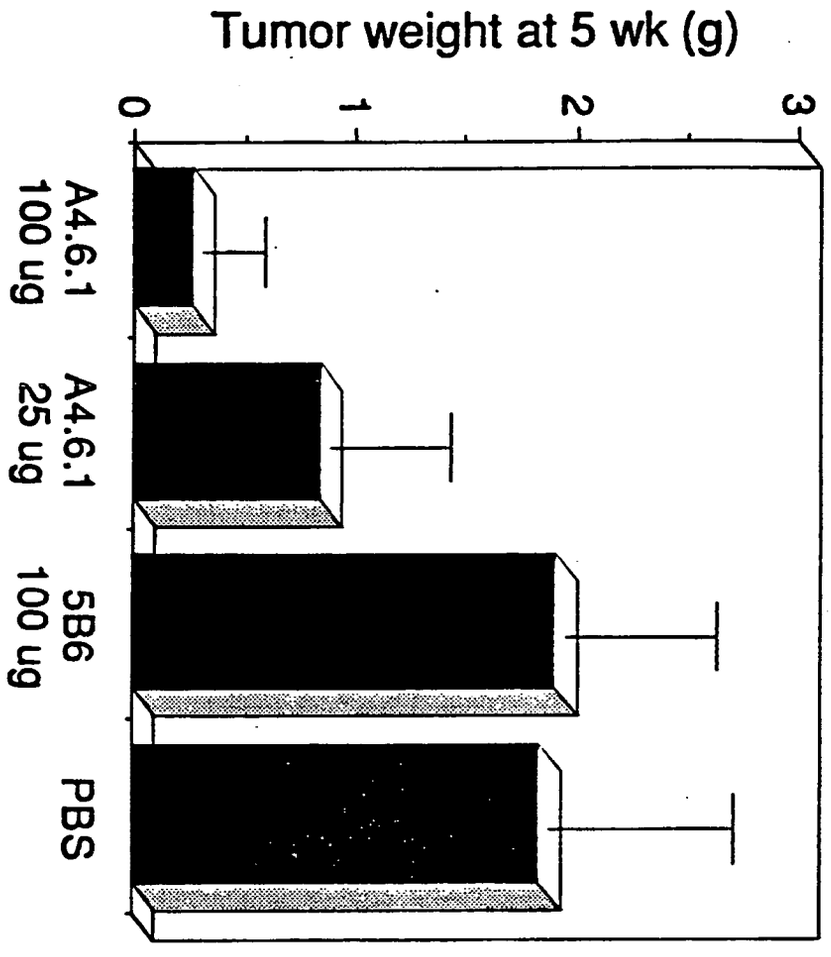


Figure 6

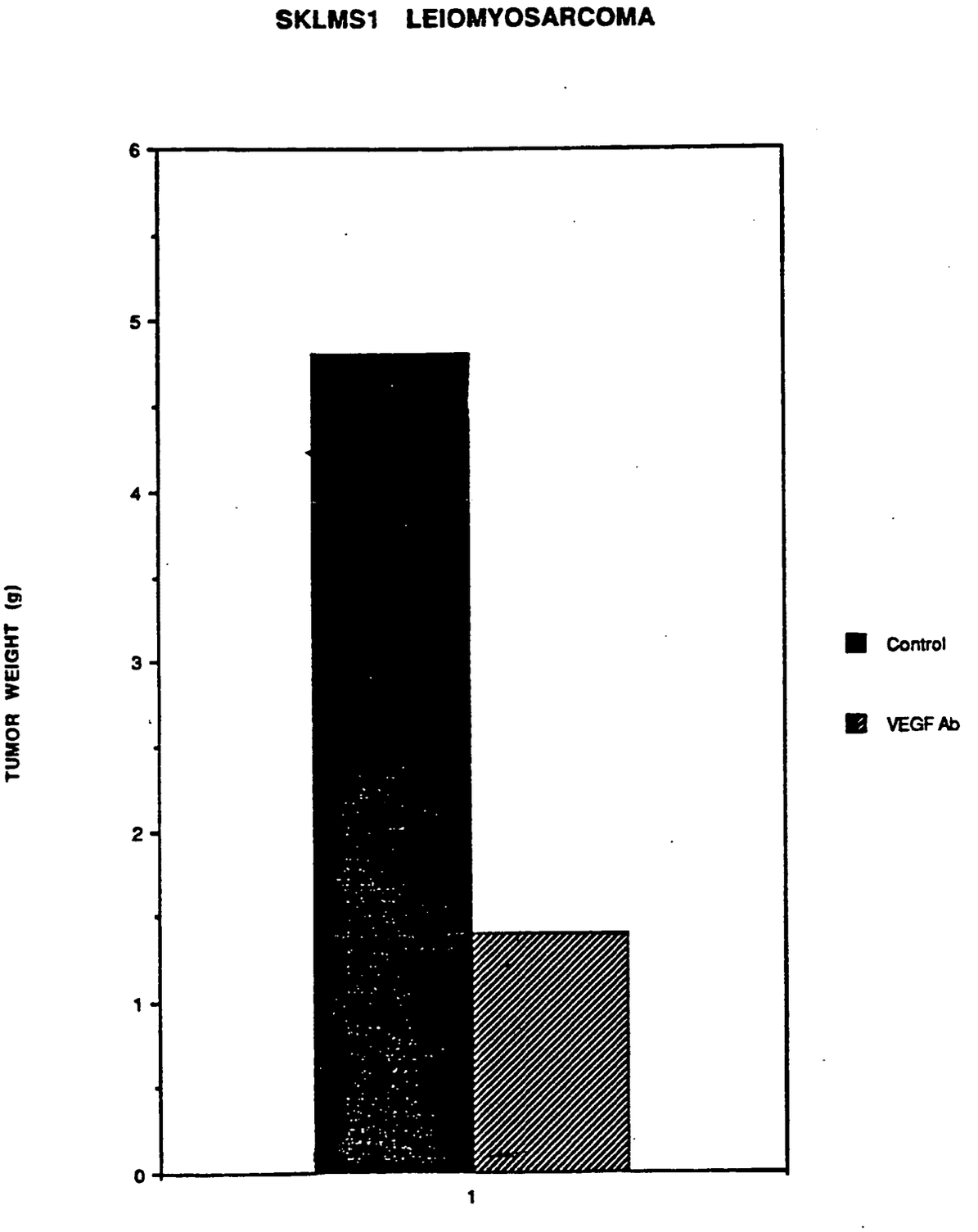
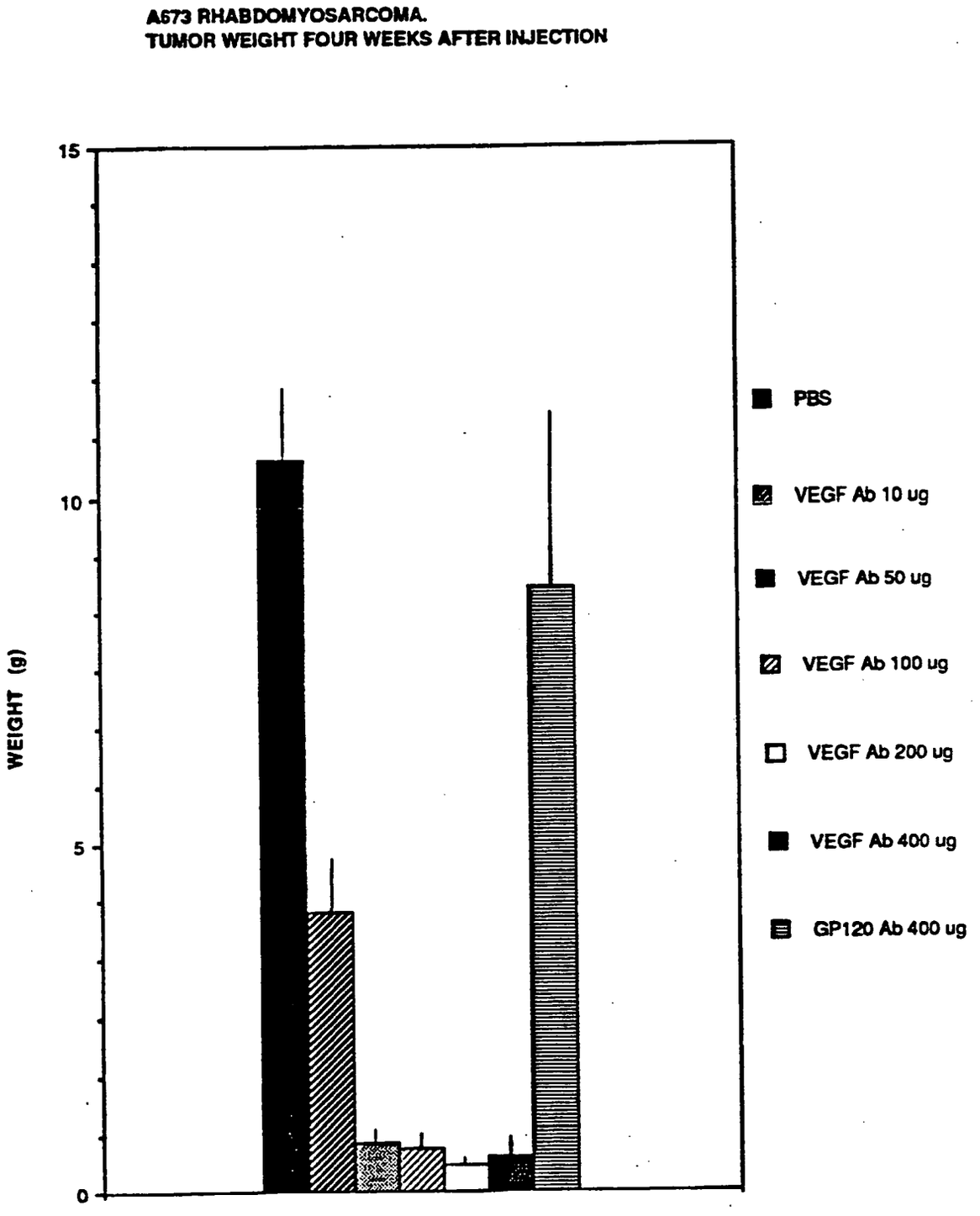


Figure 7



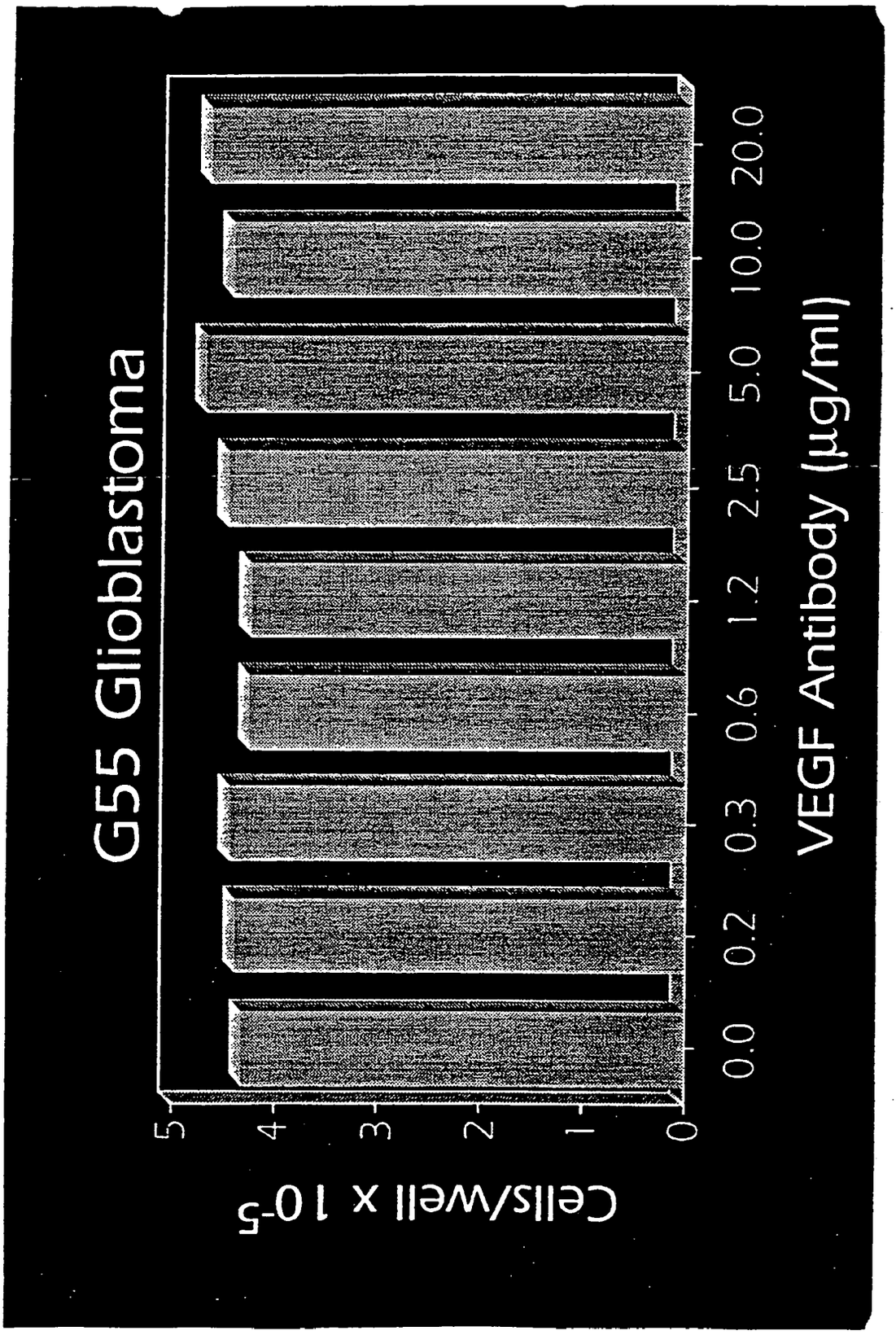


Figure 8

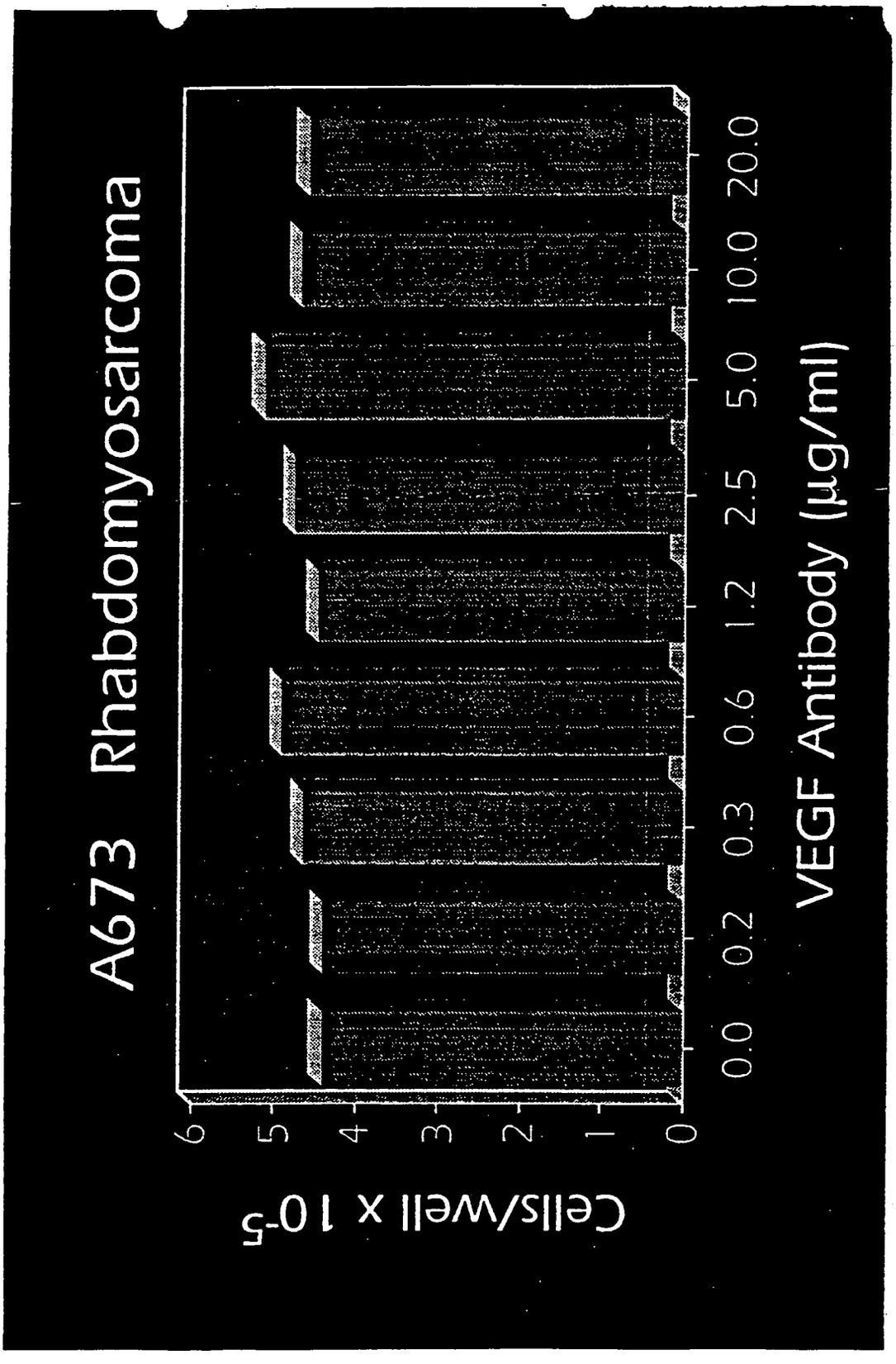


Figure 9

Figure 10

Endothelial Chemotaxis (cell number)

Sample Type	Subject #	Assay Date	Syn. Fluid	Syn. Fluid + mAB VEGF	% Suppression
Rheumatoid Syn. Fluid	318	5.7.92	5.2±0.2	2.7±0.3	48
	150	5.7.92	7.0±0.3	2.8±0.4	60
	312	5.7.92	6.7±0.4	3.7±0.3	45
	264	5.7.92	6.2±0.4	3.1±0.3	50
	267	5.7.92	5.7±0.6	4.4±0.3	23
	202	5.22.92	10.0±0.5	3.4±0.6	66
	314	5.22.92	7.5±0.3	3.1±0.6	59
	237	5.22.92	6.1±0.5	2.2±0.3	64
	206	5.22.92	6.7±0.5	2.2±0.3	67
	317	5.22.92	5.2±0.3	2.5±0.6	52
Osteoarthritis Syn. Fluid	165	6.2.92	4.0±0.3	2.8±0.4	30
	211	6.2.92	3.4±0.5	3.0±0.2	11.7
	195	6.2.92	3.5±0.2	3.3±0.3	5.7
	122	6.2.92	3.7±0.3	3.2±0.4	13.5
	16	6.2.92	4.1±0.3	3.8±0.5	7.3

Mean % Suppression for RA Fluids 53.4±4.2
Mean % Suppression for OA Fluids 13.6±3.9
Synovial fluids were diluted 1:50.

Controls:

6.2.92	PBS	3.3±0.30
	bFGF 1µg/ml	5.7±0.38
5.22.92	PBS	1.2±0.38
	bFGF 1µg/ml	7.8±0.31
5.2.92	PBS	1.3±0.18
	bFGF 1µg/ml	9.0±0.41

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 92/09218

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C07K15/00; C12P21/08; A61K39/395; A61K37/02

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C07K ; C12P ; A61K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
A	THE JOURNAL OF BIOLOGICAL CHEMISTRY vol. 264, no. 33, 25 November 1989, WASHINGTON DC, US pages 20017 - 20024 D. CONNOLLY ET AL. 'Human vascular permeability factor. Isolation from U937 cells.' see abstract see page 20021, left column, line 3 - right column, line 13 ---	1,3,5,6
A	JOURNAL OF CELLULAR BIOCHEMISTRY vol. SUPPL, no. 15F, 1991, NEW YORK, US page 251 B. LI ET AL. 'Monoclonal antibodies to recombinant human vascular endothelial growth factor (rHuVEGF).' see abstract CF 417 -----	1

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
24 JUNE 1993	08 -07- 1993
International Searching Authority	**Signature of Authorized Officer**
EUR PEAN PATENT OFFICE	NOOIJ F.J.M.

Form PCT/ISA/210 (second sheet) (January 1985)

INTERNATIONAL SEARCH REPORT

Inte .onal application No.
PCT/US92/09218

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark :Although claims 27-30 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

UNITED STATES PATENT AND TRADEMARK OFFICE

COMMISSIONER FOR PATENTS
UNITED STATES PATENT AND TRADEMARK OFFICE
WASHINGTON, D.C. 20231
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APPLICATION NUMBER	FILING/RECEIPT DATE	FIRST NAMED APPLICANT	ATTORNEY DOCKET NUMBER
09/723,752	11/27/2000	Manuel Baca	P1093P1D1

CONFIRMATION NO. 6340

FORMALITIES LETTER

OC0000000006011720

Attn: Steven X. Cui
GENENTECH, INC.
1 DNA Way
South San Francisco, CA 94080-4990

Date Mailed: 04/26/2001

NOTICE TO FILE MISSING PARTS OF NONPROVISIONAL APPLICATION

FILED UNDER 37 CFR 1.53(b)

Filing Date Granted

An application number and filing date have been accorded to this application. The item(s) indicated below, however, are missing. Applicant is given **TWO MONTHS** from the date of this Notice within which to file all required items and pay any fees required below to avoid abandonment. Extensions of time may be obtained by filing a petition accompanied by the extension fee under the provisions of 37 CFR 1.136(a).

- The statutory basic filing fee is missing.
  Applicant must submit $ **710** *to complete the basic filing fee and/or file a small entity statement claiming such status (37 CFR 1.27).*
- To avoid abandonment, a late filing fee or oath or declaration surcharge as set forth in 37 CFR 1.16(e) of $130 for a non-small entity, must be submitted with the missing items identified in this letter.
- **The balance due by applicant is $ 840.**

- This application does not contain a statement that the content of the sequence listing information recorded in computer readable form is identical to the written (on paper or compact disc) sequence listing and, where applicable, includes no new matter, as required by 37 CFR 1.821(e), 1.821(f), 1.821 (g), 1.825(b), or 1.825(d). Applicant must provide such statement. If the effective filing date is on or after September 8, 2000, see the final rulemaking notice published in the Federal Register at 65 FR 54604 (September 8, 2000) and 1238 OG 145 (September 19, 2000).
- A copy of the "Sequence Listing" in computer readable form has not been submitted as required by 37 C.F.R. 1.821(e). If the effective filing date is on or after September 8, 2000, see the final rulemaking notice published in the Federal Register at 65 FR 54604 (September 8, 2000) and 1238 OG 145 (September 19, 2000). Applicant must provide an initial computer readable form (CRF) copy of the "Sequence Listing" and a statement that the content of the sequence listing information recorded in computer readable form is identical to the written (on paper or compact disc) sequence listing and, where applicable, includes no new matter, as required by 37 CFR 1.821(e), 1.821(f), 1.821(g), 1.825 (b), or 1.825(d). If applicant desires the sequence listing in the instant application to be identical with that of another application on file in the U.S. Patent and Trademark Office, such request in accordance with 37 CFR 1.821(e) may be submitted in lieu of a new CRF.

For questions regarding compliance to these requirements, please contact:

- **For Rules Interpretation, call (703) 308-4216**
- **To Purchase PatentIn Software, call (703) 306-2600**
- **For PatentIn Software Program Help, call (703) 306-4119 or e-mail at patin21help@uspto.gov or patin3help@uspto.gov**

A copy of this notice MUST be returned with the reply.

Customer Service Center
Initial Patent Examination Division (703) 308-1202
PART 3 - OFFICE COPY

#4

B

Attorney Docket P1093P1D1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Manuel Baca et al. Serial No.: 09/723,752 Filed: November 27, 2000 For: ANTI-VEGF ANTIBODIES	Group Art Unit: To Be Assigned Examiner: To Be Assigned CERTIFICATE OF MAILING I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Assistant Commissioner of Patents, Washington, D.C. 20231 on June 26, 2001 Eileen Ly

PRELIMINARY AMENDMENT

Box Missing Parts
Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

Prior to Examination, Applicants respectfully request entry of the following Preliminary Amendment.

In the Claims:

Please cancel claims 39-42.

Please add new claims 43-59 as follows:

43. (New) A method for inhibiting VEGF-induced angiogenesis in a subject, comprising administering to said subject an effective amount of a humanized anti-VEGF antibody which binds human VEGF with a K_d value of no more than about 1×10^{-8}M.

44. (New) The method of claim 43, wherein said humanized anti-VEGF antibody which binds human VEGF with a K_d value of no more than about 1×10^{-9}M.

45. (New) The method of claim 43, wherein said subject has a tumor.

46. (New) The method of claim 45, wherein 5mg/kg of said humanized antibody inhibits at least about 50% of tumor growth in an A673 *in vivo* tumor model.

47. (New) The method of claim 43, said humanized anti-VEGF antibody having a heavy chain variable domain comprising the following hypervariable region amino acid sequences: CDRH1 (GYX_1FTX_2YGMN, wherein X_1 is T or D and X_2 is N or H; SEQ ID NO: 128), CDRH2

(WINTYTGEPTYAADFKR; SEQ ID NO:2) and CDRH3 ($YPX_1YYGX_2SHWYFDV$, wherein X_1 is Y or H and X_2 is S or T; SEQ ID NO: 129).

48. (New) The method of claim 47, said humanized anti-VEGF antibody comprising the amino acid sequence of SEQ ID NO:7.

49. (New) The method of claim 47, said humanized anti-VEGF antibody having a heavy chain variable domain comprising the following hypervariable region amino acid sequences: CDRH1 (GYTFTNYGMN; SEQ ID NO:1), CDRH2 (WINTYTGEPTYAADFKR; SEQ ID NO:2) and CDRH3 (YPHYYGSSHWYFDV; SEQ ID NO:3).

50. (New) The method of claim 43, said humanized anti-VEGF antibody having a light chain variable domain comprising the following hypervariable region amino acid sequences: CDRL1 (SASQDISNYLN; SEQ ID NO:4), CDRL2 (FTSSLHS; SEQ ID NO:5) and CDRL3 (QQYSTVPWT; SEQ ID NO:6).

51. (New) The method of claim 50, said humanized anti-VEGF antibody comprising the amino acid sequence of SEQ ID NO:8.

52. (New) The method of claim 43, said humanized anti-VEGF antibody having a heavy chain variable domain comprising the amino acid sequence of SEQ ID NO:7 and a light chain variable domain comprising the amino acid sequence of SEQ ID NO:8.

53. (New) The method of claim 43, wherein said humanized anti-VEGF antibody is a full length antibody.

54. (New) The method of claim 53, wherein said humanized anti-VEGF antibody is a human IgG.

55. (New) The method of claim 43, wherein said humanized anti-VEGF antibody is an antibody fragment.

56. (New) The method of claim 55, wherein said humanized anti-VEGF antibody is a Fab.

57. (New) The method of claim 43, wherein said subject has a retinal disease.

58. (New) The method of claim 57, wherein said retinal disease is age-related macular degeneration (AMD).

59. (New) The method of claim 58, wherein the humanized anti-VEGF antibody is administered to the subject at a dose of at least about 0.5mg/kg.

REMARKS

Claims 39-42 have been canceled, and claims 43-59 are hereby added prior to examination of the application on the merits. The amendments can find support in the specification, for example, at pages 46-48, and therefore do not add new matter. Early entry of these amendments is requested.

Respectfully submitted,

GENENTECH, INC.

Date: June 26, 2001

By: _____

Steven X. Cui
Reg. No. 44,637
Telephone No. (650) 225-8674

09157

PATENT TRADEMARK OFFICE

Document # 92549

Section

Patent Docket P1093PUD1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Manuel Baca et al. Serial No.: 09/723,752 Filed: November 27, 2000 For: ANTI-VEGF ANTIBODIES	Group Art Unit: To Be Assigned Examiner: To Be Assigned CERTIFICATE OF MAILING I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Assistant Commissioner of Patents, Washington, D.C. 20231 on June 26, 2001 Eileen Ly

RESPONSE TO NOTICE TO FILE MISSING PARTS OF APPLICATION

Box Missing Parts
Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

This is responsive to the Notice to File Missing Parts - Filing Date Granted dated April 26, 2001. Transmitted herewith are the following documents:

1. Certificate Re: Sequence Listing Response Under 37 CFR § 1.821(f) and (g).
2. Letter and Request to Use Computer-Readable Sequence Listing Under 37 CFR § 1.821(e).
3. Copy of Notice to File Missing Parts.
4. Preliminary Amendment.

Fee Calculation (37 CFR 1.16)

The fee has been calculated as follows:

CLAIMS FOR FEE CALCULATION

Number Filed					
Number Extra		Rate			Basic Fee 37 CFR 1.16(a)
					$710.00
Total Claims	17	- 20 =	0	X $18.00	$0.00

#91824

Revised (10/18/95)

Independent Claims	1	- 3 =	0	X $80.00	$0.00
—	Multiple dependent claim(s), if any			+ $270.00 ..	$0.00 .....
				Filing Fee Calculation	$710.00

Method of Payment of Fees

The Commissioner is hereby authorized to charge Deposit Account No. 07-0630 in the amount of $710.00.

The Commissioner is hereby authorized to deduct the appropriate surcharge fee of $130 associated with this communication or credit any overpayment to Deposit Account No. 07-0630. A duplicate of this sheet is enclosed.

Respectfully submitted,

GENENTECH, INC.

Date: June 26, 2001

By: Steven X. Cui

Steven X. Cui
Reg. No. 44,637
Telephone No. (650) 225-8674

09157
PATENT TRADEMARK OFFICE

#5

Patent Docket P1093P1D1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Manuel Baca et al. Serial No.: 09/723,752 Filed: November 27, 2000 For: ANTI-VEGF ANTIBODIES	Group Art Unit: Examiner: **CERTIFICATE OF MAILING** I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Assistant Commissioner of Patents, Washington, D.C. 20231 on June 26, 2001 Eileen Ly

CERTIFICATE RE: SEQUENCE LISTING

RESPONSE UNDER 37 CFR § 1.821(f) and (g)

Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

I hereby state that the Sequence Listing submitted herewith is submitted in paper copy and a computer-readable diskette, and that the information recorded in computer readable form is identical to the written sequence listing. I further state that this submission includes no new matter.

Respectfully submitted,

GENENTECH, INC.

Date: June 26, 2001

By: _____
Steven X. Cui
Reg. No. 44,637
Telephone No. (650) 225-8674

09157
PATENT TRADEMARK OFFICE

#91818

Patent Docket P1093P1D1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Manuel Baca et al. Serial No.: 09/723,752 Filed: November 27, 2000 For: ANTI-VEGF ANTIBODIES	Group Art Unit: Examiner: CERTIFICATE OF MAILING I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Assistant Commissioner of Patents, Washington, D.C. 20231 on June 26, 2001 Eileen Ly

Letter and REQUEST TO USE COMPUTER-READABLE SEQUENCE LISTING

UNDER 37 CFR §1.821(e)

Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

Applicants respectfully request that the compliant computer-readable Sequence Listing filed in application Serial No. 08/908,469 be used as the computer-readable Sequence Listing for the present, above-identified application.

The paper copy of the Sequence Listing filed in the present application is identical to the computer-readable copy of the Sequence Listing filed in the application Serial No. 08/908,469.

Respectfully submitted,

GENENTECH, INC.

Date: June 26, 2001

By: Steven X. Cui

Steven X. Cui
Reg. No. 44,637
Telephone No. (650) 225-8674

09157
PATENT TRADEMARK OFFICE

#91819

#6

UNITED STATES PATENT AND TRADEMARK OFFICE

COMMISSIONER FOR PATENTS
UNITED STATES PATENT AND TRADEMARK OFFICE
WASHINGTON, D.C. 20231
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APPLICATION NUMBER	FILING/RECEIPT DATE	FIRST NAMED APPLICANT	ATTORNEY DOCKET NUMBER
09/723,752	11/27/2000	Manuel Baca	P1093P1D1

CONFIRMATION NO. 6340

FORMALITIES LETTER


OC000000006011720

Attn: Steven X. Cui
GENENTECH, INC.
1 DNA Way
South San Francisco, CA 94080-4990

Date Mailed: 04/26/2001

NOTICE TO FILE MISSING PARTS OF NONPROVISIONAL APPLICATION

FILED UNDER 37 CFR 1.53(b)

Filing Date Granted

An application number and filing date have been accorded to this application. The item(s) indicated below, however, are missing. Applicant is given **TWO MONTHS** from the date of this Notice within which to file all required items and pay any fees required below to avoid abandonment. Extensions of time may be obtained by filing a petition accompanied by the extension fee under the provisions of 37 CFR 1.136(a).

- The statutory basic filing fee is missing.
  Applicant must submit $ **710** *to complete the basic filing fee and/or file a small entity statement claiming such status (37 CFR 1.27).*
- To avoid abandonment, a late filing fee or oath or declaration surcharge as set forth in 37 CFR 1.16(e) of $130 for a non-small entity, must be submitted with the missing items identified in this letter.
- **The balance due by applicant is $ 840.**

- This application does not contain a statement that the content of the sequence listing information recorded in computer readable form is identical to the written (on paper or compact disc) sequence listing and, where applicable, includes no new matter, as required by 37 CFR 1.821(e), 1.821(f), 1.821 (g), 1.825(b), or 1.825(d). Applicant must provide such statement. If the effective filing date is on or after September 8, 2000, see the final rulemaking notice published in the Federal Register at 65 FR 54604 (September 8, 2000) and 1238 OG 145 (September 19, 2000).
- A copy of the "Sequence Listing" in computer readable form has not been submitted as required by 37 C.F.R. 1.821(e). If the effective filing date is on or after September 8, 2000, see the final rulemaking notice published in the Federal Register at 65 FR 54604 (September 8, 2000) and 1238 OG 145 (September 19, 2000). Applicant must provide an initial computer readable form (CRF) copy of the "Sequence Listing" and a statement that the content of the sequence listing information recorded in computer readable form is identical to the written (on paper or compact disc) sequence listing and, where applicable, includes no new matter, as required by 37 CFR 1.821(e), 1.821(f), 1.821(g), 1.825 (b), or 1.825(d). If applicant desires the sequence listing in the instant application to be identical with that of another application on file in the U.S. Patent and Trademark Office, such request in accordance with 37 CFR 1.821(e) may be submitted in lieu of a new CRF.

07/09/2001 EEKUBAY1 00000110 070630 09723752

01 FC:101 710.00 CH
02 FC:105 130.00 CH

For questions regarding compliance to these requirements, please contact:

- **For Rules Interpretation, call (703) 308-4216**
- **To Purchase PatentIn Software, call (703) 306-2600**
- **For PatentIn Software Program Help, call (703) 306-4119 or e-mail at patin21help@uspto.gov or patin3help@uspto.gov**

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Customer Service Center
Initial Patent Examination Division (703) 308-1202
PART 2 - COPY TO BE RETURNED WITH RESPONSE

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1202

#7

OIPE

RAW SEQUENCE LISTING DATE: 12/14/2001
PATENT APPLICATION: US/09/723,752 TIME: 15:32:45

Input Set : N:\Crf3\RULE60\09723752.txt
Output Set: N:\CRF3\12142001\I723752.raw

SEQUENCE LISTING

W--> 3 SEQUENCE LISTING

ENTERED

5 (1) GENERAL INFORMATION:
7 (i) APPLICANT: Baca, Manuel
8 Wells, James A.
9 Presta, Leonard G.
10 Lowman, Henry B.
11 Chen, Yvonne M.
13 (ii) TITLE OF INVENTION: ANTI-VEGF ANTIBODIES
15 (iii) NUMBER OF SEQUENCES: 131
17 (iv) CORRESPONDENCE ADDRESS:
18 (A) ADDRESSEE: Genentech, Inc.
19 (B) STREET: 1 DNA Way
20 (C) CITY: South San Francisco
21 (D) STATE: California
22 (E) COUNTRY: USA
23 (F) ZIP: 94080
25 (v) COMPUTER READABLE FORM:
26 (A) MEDIUM TYPE: 3.5 inch, 1.44 Mb floppy disk
27 (B) COMPUTER: IBM PC compatible
28 (C) OPERATING SYSTEM: PC-DOS/MS-DOS
29 (D) SOFTWARE: WinPatin (Genentech)
31 (vi) CURRENT APPLICATION DATA:
C--> 32 **(A) APPLICATION NUMBER: US/09/723,752**
C--> 33 **(B) FILING DATE: 27-Nov-2000**
34 (C) CLASSIFICATION:
36 (vii) PRIOR APPLICATION DATA:
37 (A) APPLICATION NUMBER: 08/908,469
38 (B) FILING DATE: 1997-08-06
40 (viii) ATTORNEY/AGENT INFORMATION:
41 (A) NAME: Cui, Steven X.
42 (B) REGISTRATION NUMBER: 44,637
43 (C) REFERENCE/DOCKET NUMBER: P1093P1
45 (ix) TELECOMMUNICATION INFORMATION:
46 (A) TELEPHONE: 650/225-8674
47 (B) TELEFAX: 650/952-9881
48 (2) INFORMATION FOR SEQ ID NO: 1:
50 (i) SEQUENCE CHARACTERISTICS:
51 (A) LENGTH: 10 amino acids
52 (B) TYPE: Amino Acid
53 (D) TOPOLOGY: Linear
55 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:
57 Gly Tyr Thr Phe Thr Asn Tyr Gly Met Asn
58 1 5 10
60 (2) INFORMATION FOR SEQ ID NO: 2:
62 (i) SEQUENCE CHARACTERISTICS:
63 (A) LENGTH: 17 amino acids

RAW SEQUENCE LISTING DATE: 12/14/2001
PATENT APPLICATION: US/09/723,752 TIME: 15:32:46

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Output Set: N:\CRF3\12142001\I723752.raw

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64          (B) TYPE: Amino Acid
65          (D) TOPOLOGY: Linear
67     (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:
69  Trp Ile Asn Thr Tyr Thr Gly Glu Pro Thr Tyr Ala Ala Asp Phe
70    1               5                  10                  15
72  Lys Arg
75 (2) INFORMATION FOR SEQ ID NO: 3:
77      (i) SEQUENCE CHARACTERISTICS:
78          (A) LENGTH: 14 amino acids
79          (B) TYPE: Amino Acid
80          (D) TOPOLOGY: Linear
82     (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:
84  Tyr Pro His Tyr Tyr Gly Ser Ser His Trp Tyr Phe Asp Val
85    1               5                  10
87 (2) INFORMATION FOR SEQ ID NO: 4:
89      (i) SEQUENCE CHARACTERISTICS:
90          (A) LENGTH: 11 amino acids
91          (B) TYPE: Amino Acid
92          (D) TOPOLOGY: Linear
94     (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:
96  Ser Ala Ser Gln Asp Ile Ser Asn Tyr Leu Asn
97    1               5                  10
99 (2) INFORMATION FOR SEQ ID NO: 5:
101     (i) SEQUENCE CHARACTERISTICS:
102         (A) LENGTH: 7 amino acids
103         (B) TYPE: Amino Acid
104         (D) TOPOLOGY: Linear
106    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:
108  Phe Thr Ser Ser Leu His Ser
109    1               5
111 (2) INFORMATION FOR SEQ ID NO: 6:
113     (i) SEQUENCE CHARACTERISTICS:
114         (A) LENGTH: 9 amino acids
115         (B) TYPE: Amino Acid
116         (D) TOPOLOGY: Linear
118    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:
120  Gln Gln Tyr Ser Thr Val Pro Trp Thr
121    1               5
123 (2) INFORMATION FOR SEQ ID NO: 7:
125     (i) SEQUENCE CHARACTERISTICS:
126         (A) LENGTH: 123 amino acids
127         (B) TYPE: Amino Acid
128         (D) TOPOLOGY: Linear
130    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:
132  Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
133    1               5                  10                  15
135  Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr
136                   20                  25                  30
138  Asn Tyr Gly Met Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
```

RAW SEQUENCE LISTING DATE: 12/14/2001
PATENT APPLICATION: US/09/723,752 TIME: 15:32:46

Input Set : N:\Crf3\RULE60\09723752.txt
Output Set: N:\CRF3\12142001\I723752.raw

```
                    35                  40                  45
 Glu Trp Val Gly Trp Ile Asn Thr Tyr Thr Gly Glu Pro Thr Tyr
                    50                  55                  60
 Ala Ala Asp Phe Lys Arg Arg Phe Thr Phe Ser Leu Asp Thr Ser
                    65                  70                  75
 Lys Ser Thr Ala Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
                    80                  85                  90
 Thr Ala Val Tyr Tyr Cys Ala Lys Tyr Pro His Tyr Tyr Gly Ser
                    95                 100                 105
 Ser His Trp Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu Val Thr
                   110                 115                 120
 Val Ser Ser
(2) INFORMATION FOR SEQ ID NO: 8:
     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 108 amino acids
          (B) TYPE: Amino Acid
          (D) TOPOLOGY: Linear
    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:
 Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
   1               5                  10                  15
 Gly Asp Arg Val Thr Ile Thr Cys Ser Ala Ser Gln Asp Ile Ser
                    20                  25                  30
 Asn Tyr Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
                    35                  40                  45
 Val Leu Ile Tyr Phe Thr Ser Ser Leu His Ser Gly Val Pro Ser
                    50                  55                  60
 Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
                    65                  70                  75
 Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
                    80                  85                  90
 Tyr Ser Thr Val Pro Trp Thr Phe Gly Gln Gly Thr Lys Val Glu
                    95                 100                 105
 Ile Lys Arg
(2) INFORMATION FOR SEQ ID NO: 9:
     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 123 amino acids
          (B) TYPE: Amino Acid
          (D) TOPOLOGY: Linear
    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:
 Glu Ile Gln Leu Val Gln Ser Gly Pro Glu Leu Lys Gln Pro Gly
   1               5                  10                  15
 Glu Thr Val Arg Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr
                    20                  25                  30
 Asn Tyr Gly Met Asn Trp Val Lys Gln Ala Pro Gly Lys Gly Leu
                    35                  40                  45
 Lys Trp Met Gly Trp Ile Asn Thr Tyr Thr Gly Glu Pro Thr Tyr
                    50                  55                  60
 Ala Ala Asp Phe Lys Arg Arg Phe Thr Phe Ser Leu Glu Thr Ser
                    65                  70                  75
```

RAW SEQUENCE LISTING DATE: 12/14/2001
PATENT APPLICATION: US/09/723,752 TIME: 15:32:46

Input Set : N:\Crf3\RULE60\09723752.txt
Output Set: N:\CRF3\12142001\I723752.raw

```
216  Ala Ser Thr Ala Tyr Leu Gln Ile Ser Asn Leu Lys Asn Asp Asp
217                  80                  85                  90
219  Thr Ala Thr Tyr Phe Cys Ala Lys Tyr Pro His Tyr Tyr Gly Ser
220                  95                 100                 105
222  Ser His Trp Tyr Phe Asp Val Trp Gly Ala Gly Thr Thr Val Thr
223                 110                 115                 120
225  Val Ser Ser
228 (2) INFORMATION FOR SEQ ID NO: 10:
230      (i) SEQUENCE CHARACTERISTICS:
231           (A) LENGTH: 108 amino acids
232           (B) TYPE: Amino Acid
233           (D) TOPOLOGY: Linear
235     (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:
237  Asp Ile Gln Met Thr Gln Thr Thr Ser Ser Leu Ser Ala Ser Leu
238    1               5                  10                  15
240  Gly Asp Arg Val Ile Ile Ser Cys Ser Ala Ser Gln Asp Ile Ser
241                  20                  25                  30
243  Asn Tyr Leu Asn Trp Tyr Gln Gln Lys Pro Asp Gly Thr Val Lys
244                  35                  40                  45
246  Val Leu Ile Tyr Phe Thr Ser Ser Leu His Ser Gly Val Pro Ser
247                  50                  55                  60
249  Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Tyr Ser Leu Thr Ile
250                  65                  70                  75
252  Ser Asn Leu Glu Pro Glu Asp Ile Ala Thr Tyr Tyr Cys Gln Gln
253                  80                  85                  90
255  Tyr Ser Thr Val Pro Trp Thr Phe Gly Gly Gly Thr Lys Leu Glu
256                  95                 100                 105
258  Ile Lys Arg
261 (2) INFORMATION FOR SEQ ID NO: 11:
263      (i) SEQUENCE CHARACTERISTICS:
264           (A) LENGTH: 113 amino acids
265           (B) TYPE: Amino Acid
266           (D) TOPOLOGY: Linear
268     (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:
270  Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
271    1               5                  10                  15
273  Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser
274                  20                  25                  30
276  Ser Tyr Ala Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
277                  35                  40                  45
279  Glu Trp Val Ser Val Ile Ser Gly Asp Gly Gly Ser Thr Tyr Tyr
280                  50                  55                  60
282  Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser
283                  65                  70                  75
285  Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
286                  80                  85                  90
288  Thr Ala Val Tyr Tyr Cys Ala Arg Gly Phe Asp Tyr Trp Gly Gln
289                  95                 100                 105
291  Gly Thr Leu Val Thr Val Ser Ser
```

RAW SEQUENCE LISTING DATE: 12/14/2001
PATENT APPLICATION: US/09/723,752 TIME: 15:32:46

Input Set : N:\Crf3\RULE60\09723752.txt
Output Set: N:\CRF3\12142001\I723752.raw

292 110
294 (2) INFORMATION FOR SEQ ID NO: 12:
296 (i) SEQUENCE CHARACTERISTICS:
297 (A) LENGTH: 108 amino acids
298 (B) TYPE: Amino Acid
299 (D) TOPOLOGY: Linear
301 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:
303 Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
304 1 5 10 15
306 Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Ser Ile Ser
307 20 25 30
309 Asn Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
310 35 40 45
312 Leu Leu Ile Tyr Ala Ala Ser Ser Leu Glu Ser Gly Val Pro Ser
313 50 55 60
315 Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
316 65 70 75
318 Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
319 80 85 90
321 Tyr Asn Ser Leu Pro Trp Thr Phe Gly Gln Gly Thr Lys Val Glu
322 95 100 105
324 Ile Lys Arg
327 (2) INFORMATION FOR SEQ ID NO: 13:
329 (i) SEQUENCE CHARACTERISTICS:
330 (A) LENGTH: 107 amino acids
331 (B) TYPE: Amino Acid
332 (D) TOPOLOGY: Linear
334 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:
336 Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
337 1 5 10 15
339 Gly Asp Arg Val Thr Ile Thr Cys Ser Ala Ser Gln Asp Ile Ser
340 20 25 30
342 Asn Tyr Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
343 35 40 45
345 Leu Leu Ile Tyr Phe Thr Ser Ser Leu His Ser Gly Val Pro Ser
346 50 55 60
348 Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
349 65 70 75
351 Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
352 80 85 90
354 Tyr Ser Thr Val Pro Trp Thr Phe Gly Gln Gly Thr Lys Val Glu
355 95 100 105
357 Ile Lys
360 (2) INFORMATION FOR SEQ ID NO: 14:
362 (i) SEQUENCE CHARACTERISTICS:
363 (A) LENGTH: 123 amino acids
364 (B) TYPE: Amino Acid
365 (D) TOPOLOGY: Linear
367 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

VERIFICATION SUMMARY DATE: 12/14/2001
PATENT APPLICATION: **US/09/723,752** TIME: 15:32:47

Input Set : **N:\Crf3\RULE60\09723752.txt**
Output Set: **N:\CRF3\12142001\I723752.raw**

L:3 M:244 W: Invalid beginning of sequence listing, Data=[SEQUENCE LISTING], Duplicate Sequence Listing Title!
L:32 M:220 C: Keyword misspelled or invalid format, [(A) APPLICATION NUMBER:]
L:33 M:220 C: Keyword misspelled or invalid format, [(B) FILING DATE:]
L:2469 M:341 W: (46) "n" or "Xaa" used, for SEQ ID#:119
L:2481 M:341 W: (46) "n" or "Xaa" used, for SEQ ID#:120
L:2496 M:341 W: (46) "n" or "Xaa" used, for SEQ ID#:121
L:2508 M:341 W: (46) "n" or "Xaa" used, for SEQ ID#:122
L:2520 M:341 W: (46) "n" or "Xaa" used, for SEQ ID#:123
L:2544 M:341 W: (46) "n" or "Xaa" used, for SEQ ID#:125
L:2556 M:341 W: (46) "n" or "Xaa" used, for SEQ ID#:126
L:2592 M:341 W: (46) "n" or "Xaa" used, for SEQ ID#:127
L:2595 M:341 W: (46) "n" or "Xaa" used, for SEQ ID#:127
L:2607 M:341 W: (46) "n" or "Xaa" used, for SEQ ID#:127
L:2649 M:341 W: (46) "n" or "Xaa" used, for SEQ ID#:130
L:2661 M:341 W: (46) "n" or "Xaa" used, for SEQ ID#:131

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UNITED STATES PATENT AND TRADEMARK OFFICE
WASHINGTON, D.C. 20231
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APPLICATION NUMBER	FILING DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
09/723,752	11/27/2000	Manuel Baca	P1093P1D1

CONFIRMATION NO. 6340

WITHDRAWAL NOTICE

OC000000007223681

Attn: Steven X. Cui
GENENTECH, INC.
1 DNA Way
South San Francisco, CA 94080-4990

Date Mailed: 12/26/2001

WITHDRAWAL OF PREVIOUSLY SENT NOTICE

The Notice mailed on 04/26/2001 was sent in error and is hereby withdrawn. A corrected Notice is enclosed. The time period for reply runs from the mail date of the corrected Notice. We apologize for any inconvenience this caused.

*A copy of this notice **MUST** be returned with the reply.*

Customer Service Center
Initial Patent Examination Division (703) 308-1202
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WASHINGTON, D.C. 20231
www.uspto.gov

APPLICATION NUMBER	FILING/RECEIPT DATE	FIRST NAMED APPLICANT	ATTORNEY DOCKET NUMBER
09/723,752	11/27/2000	Manuel Baca	P1093P1D1

CONFIRMATION NO. 6340

FORMALITIES LETTER

OC000000007228019

Attn: Steven X. Cui
GENENTECH, INC.
1 DNA Way
South San Francisco, CA 94080-4990

Date Mailed: 12/26/2001

NOTICE TO COMPLY WITH REQUIREMENTS FOR PATENT APPLICATIONS CONTAINING NUCLEOTIDE SEQUENCE AND/OR AMINO ACID SEQUENCE DISCLOSURES

Applicant is given **TWO MONTHS FROM THE DATE OF THIS NOTICE** within which to file the items indicated below to avoid abandonment. Extensions of time may be obtained under the provisions of 37 CFR 1.136(a).

- This application does not contain, as a separate part of the disclosure on paper copy, a "Sequence Listing" as required by 37 C.F.R. 1.821(c) Applicant must provide an initial paper or compact disc copy of the "Sequence Listing", as well as an amendment directing its entry into the application and a statement that the content of the sequence listing information recorded in computer readable form is identical to the written (on paper or compact disc) sequence listing and, where applicable, includes no new matter, as required by 37 CFR 1.821(e), 1.821(f), 1.821(g), 1.825(b), or 1.825(d). If the effective filing date is on or after September 8, 2000, see the final rulemaking notice published in the Federal Register at 65 FR 54604 (September 8, 2000) and 1238 OG 145 (September 19, 2000).

For questions regarding compliance to these requirements, please contact:

- **For Rules Interpretation, call (703) 308-4216**
- **To Purchase PatentIn Software, call (703) 306-2600**
- **For PatentIn Software Program Help, call (703) 306-4119 or e-mail at patin21help@uspto.gov or patin3help@uspto.gov**

A copy of this notice MUST be returned with the reply.

Customer Service Center
Initial Patent Examination Division (703) 308-1202

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#9

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Baca, Manuel
Wells, James A.
Presta, Leonard G.
Lowman, Henry B.
Chen, Yvonne M.

(ii) TITLE OF INVENTION: ANTI-VEGF ANTIBODIES

(iii) NUMBER OF SEQUENCES: 131

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Genentech, Inc.
(B) STREET: 1 DNA Way
(C) CITY: South San Francisco
(D) STATE: California
(E) COUNTRY: USA
(F) ZIP: 94080

Ins C4

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: 3.5 inch, 1.44 Mb floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: WinPatin (Genentech)

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: 08/908,469
(B) FILING DATE: 06-Aug-1997
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 08/833,504
(B) FILING DATE: 07-APR-1997

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Cui, Steven X.
(B) REGISTRATION NUMBER: 44,637
(C) REFERENCE/DOCKET NUMBER: P1093P1

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 650/225-8674
(B) TELEFAX: 650/952-9881

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Gly Tyr Thr Phe Thr Asn Tyr Gly Met Asn
1 5 10

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Trp Ile Asn Thr Tyr Thr Gly Glu Pro Thr Tyr Ala Ala Asp Phe
1 5 10 15

Lys Arg

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Tyr Pro His Tyr Tyr Gly Ser Ser His Trp Tyr Phe Asp Val
1 5 10

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Ser Ala Ser Gln Asp Ile Ser Asn Tyr Leu Asn
1 5 10

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Phe Thr Ser Ser Leu His Ser
1 5

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Gln Gln Tyr Ser Thr Val Pro Trp Thr
1 5

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 123 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
1 5 10 15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr
20 25 30

Asn Tyr Gly Met Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
35 40 45

Glu Trp Val Gly Trp Ile Asn Thr Tyr Thr Gly Glu Pro Thr Tyr
50 55 60

Ala Ala Asp Phe Lys Arg Arg Phe Thr Phe Ser Leu Asp Thr Ser
65 70 75

Lys Ser Thr Ala Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
80 85 90

Thr Ala Val Tyr Tyr Cys Ala Lys Tyr Pro His Tyr Tyr Gly Ser
95 100 105

Ser His Trp Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu Val Thr
110 115 120

Val Ser Ser

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 108 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
1 5 10 15

Gly Asp Arg Val Thr Ile Thr Cys Ser Ala Ser Gln Asp Ile Ser
20 25 30

Asn Tyr Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
35 40 45

Val Leu Ile Tyr Phe Thr Ser Ser Leu His Ser Gly Val Pro Ser
50 55 60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
65 70 75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
80 85 90

Tyr Ser Thr Val Pro Trp Thr Phe Gly Gln Gly Thr Lys Val Glu
95 100 105

Ile Lys Arg

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 123 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Glu Ile Gln Leu Val Gln Ser Gly Pro Glu Leu Lys Gln Pro Gly
1 5 10 15

Glu Thr Val Arg Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr
20 25 30

Asn Tyr Gly Met Asn Trp Val Lys Gln Ala Pro Gly Lys Gly Leu
35 40 45

Lys Trp Met Gly Trp Ile Asn Thr Tyr Thr Gly Glu Pro Thr Tyr
50 55 60

Ala Ala Asp Phe Lys Arg Arg Phe Thr Phe Ser Leu Glu Thr Ser
65 70 75

Ala Ser Thr Ala Tyr Leu Gln Ile Ser Asn Leu Lys Asn Asp Asp
80 85 90

Thr Ala Thr Tyr Phe Cys Ala Lys Tyr Pro His Tyr Tyr Gly Ser
95 100 105

Ser His Trp Tyr Phe Asp Val Trp Gly Ala Gly Thr Thr Val Thr
110 115 120

Val Ser Ser

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 108 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
Asp Ile Gln Met Thr Gln Thr Thr Ser Ser Leu Ser Ala Ser Leu
 1               5                  10                  15

Gly Asp Arg Val Ile Ile Ser Cys Ser Ala Ser Gln Asp Ile Ser
            20                  25                  30

Asn Tyr Leu Asn Trp Tyr Gln Gln Lys Pro Asp Gly Thr Val Lys
            35                  40                  45

Val Leu Ile Tyr Phe Thr Ser Ser Leu His Ser Gly Val Pro Ser
            50                  55                  60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Tyr Ser Leu Thr Ile
            65                  70                  75

Ser Asn Leu Glu Pro Glu Asp Ile Ala Thr Tyr Tyr Cys Gln Gln
            80                  85                  90

Tyr Ser Thr Val Pro Trp Thr Phe Gly Gly Gly Thr Lys Leu Glu
            95                 100                 105

Ile Lys Arg
```

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 113 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```
Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
 1               5                  10                  15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser
            20                  25                  30

Ser Tyr Ala Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
            35                  40                  45

Glu Trp Val Ser Val Ile Ser Gly Asp Gly Gly Ser Thr Tyr Tyr
            50                  55                  60

Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser
            65                  70                  75

Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
            80                  85                  90

Thr Ala Val Tyr Tyr Cys Ala Arg Gly Phe Asp Tyr Trp Gly Gln
            95                 100                 105

Gly Thr Leu Val Thr Val Ser Ser
           110
```

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 108 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
 1               5                  10                  15

Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Ser Ile Ser
            20                  25                  30

Asn Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
            35                  40                  45

Leu Leu Ile Tyr Ala Ala Ser Ser Leu Glu Ser Gly Val Pro Ser
            50                  55                  60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
            65                  70                  75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
            80                  85                  90

Tyr Asn Ser Leu Pro Trp Thr Phe Gly Gln Gly Thr Lys Val Glu
            95                 100                 105

Ile Lys Arg
```

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 107 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

```
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
 1               5                  10                  15

Gly Asp Arg Val Thr Ile Thr Cys Ser Ala Ser Gln Asp Ile Ser
            20                  25                  30

Asn Tyr Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
            35                  40                  45

Leu Leu Ile Tyr Phe Thr Ser Ser Leu His Ser Gly Val Pro Ser
            50                  55                  60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
            65                  70                  75
```

```
Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
                80                  85                  90

Tyr Ser Thr Val Pro Trp Thr Phe Gly Gln Gly Thr Lys Val Glu
                95                 100                 105

Ile Lys
```

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 123 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

```
Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
 1               5                  10                  15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr
                20                  25                  30

Asn Tyr Gly Met Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
                35                  40                  45

Glu Trp Val Gly Trp Ile Asn Thr Tyr Thr Gly Glu Pro Thr Tyr
                50                  55                  60

Ala Ala Asp Phe Lys Arg Arg Phe Thr Ile Ser Arg Asp Asn Ser
                65                  70                  75

Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
                80                  85                  90

Thr Ala Val Tyr Tyr Cys Ala Arg Tyr Pro His Tyr Tyr Gly Ser
                95                 100                 105

Ser His Trp Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu Val Thr
               110                 115                 120

Val Ser Ser
```

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 107 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

```
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
 1               5                  10                  15
```

```
Gly Asp Arg Val Thr Ile Thr Cys Ser Ala Ser Gln Asp Ile Ser
                20                  25                  30

Asn Tyr Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
                35                  40                  45

Leu Leu Ile Tyr Phe Thr Ser Ser Leu His Ser Gly Val Pro Ser
                50                  55                  60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Tyr Thr Leu Thr Ile
                65                  70                  75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
                80                  85                  90

Tyr Ser Thr Val Pro Trp Thr Phe Gly Gln Gly Thr Lys Val Glu
                95                  100                 105

Ile Lys
```

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 123 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

```
Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
  1               5                  10                  15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr
                20                  25                  30

Asn Tyr Gly Met Asn Trp Ile Arg Gln Ala Pro Gly Lys Gly Leu
                35                  40                  45

Glu Trp Val Gly Trp Ile Asn Thr Tyr Thr Gly Glu Pro Thr Tyr
                50                  55                  60

Ala Ala Asp Phe Lys Arg Arg Phe Thr Ile Ser Leu Asp Thr Ser
                65                  70                  75

Ala Ser Thr Val Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
                80                  85                  90

Thr Ala Val Tyr Tyr Cys Ala Lys Tyr Pro His Tyr Tyr Gly Ser
                95                  100                 105

Ser His Trp Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu Val Thr
               110                 115                 120

Val Ser Ser
```

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Pro Lys Asn Ser Ser Met Ile Ser Asn Thr Pro
1 5 10

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

His Gln Ser Leu Gly Thr Gln
1 5

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

His Gln Asn Leu Ser Asp Gly Lys
1 5

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

His Gln Asn Ile Ser Asp Gly Lys
1 5

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Val Ile Ser Ser His Leu Gly Gln
1 5

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 66 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

GATTTCAAAC GTCGTNYTAC TWTTTCTAGA GACAACTCCA AAAACACABY 50

TTACCTGCAG ATGAAC 66

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 66 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

GATTTCAAAC GTCGTNYTAC TWTTTCTTTA GACACCTCCG CAAGCACABY 50

TTACCTGCAG ATGAAC 66

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

AGCCTGCGCG CTGAGGACAC TGCCGTCTAT TACTGTDYAA RGTACCCCCA 50

CTATTATGGG 60

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

CTCAGCGCGC AGGCTGTTCA TCTGCAGGTA 30

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

GCTGATATCC AGTTGACCCA GTCCCCG 27

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

TCTGGGACGG ATTACACTCT GACCATC 27

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 75 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

CGTTTGTCCT GTGCARYTTC TGGCTATACC TTCACCAACT ATGGTATGAA 50

CTGGRTCCGT CAGGCCCCGG GTAAG 75

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

GATATCCAGT TGACCCAGTC CCCG 24

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs

(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

GCTCCGAAAG TACTGATTTA C 21

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 54 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

CGTCGTTTCA CTTTTTCTGC AGACACCTCC AGCAACACAG TATACCTGCA 50

GATG 54

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

CTATTACTGT GCAAAGTACC CCCAC 25

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

GGGACGGATT TCACTCTGAC CATC 24

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

GGTATGAACT GGGTCCGTCA GGCCCC 26

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 57 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

CGTCGTTTCA CTTTTTCTTT AGACACCTCC AAAAGCACAG CATACCTGCA 50

GATGAAC 57

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 53 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

GGGTCACCAT CACCTGCTAA GCATAATAAT AATAAAGCAA CTATTTAAAC 50

TGG 53

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 52 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

GCGCAAGTCA GGATATTTAA TAATAATAAT AATGGTATCA ACAGAAACCA 50

GG 52

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 48 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

GTCTATTACT GTGCAAAGTA ATAACACTAA TAAGGGAGCA GCCACTGG 48

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 49 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

GGTACCCCCA CTATTATTAA TAATAATAAT GGTATTTCGA CGTCTGGGG 49

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 53 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

CACTATTATG GGAGCAGCCA CTAATAATAA TAAGTCTGGG TCAAGGAACC 50

CTG 53

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 53 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

TCCTGTGCAG CTTCTGGCTA ATAATTCTAA TAATAAGGTA TGAACTGGGT 50

CCG 53

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 52 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

GAATGGGTTG GATGGATTAA CTAATAATAA GGTTAACCGA CCTATGCTGC 50

GG 52

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 49 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

CTGTGCAAAG TACCCGTAAT ATTAATAATA ATAACACTGG TATTTCGAC 49

(2) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 48 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

CGTTTCACTT TTTCTTAAGA CTAATCCAAA TAAACAGCAT ACCTGCAG 48

(2) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 46 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

GAATGGGTTG GATGGATTTA ATAATAATAA GGTGAACCGA CCTATG 46

(2) INFORMATION FOR SEQ ID NO:46:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 53 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

GGGTCACCAT CACCTGCNNS GCANNSNNSN NSNNSAGCAA CTATTTAAAC 50

TGG 53

(2) INFORMATION FOR SEQ ID NO:47:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 52 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

GCGCAAGTCA GGATATTNNS NNSNNSNNSN NSTGGTATCA ACAGAAACCA 50

GG 52

(2) INFORMATION FOR SEQ ID NO:48:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 48 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

GTCTATTACT GTGCAAAGNN SNNSCACNNS NNSGGGAGCA GCCACTGG 48

(2) INFORMATION FOR SEQ ID NO:49:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 49 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

GGTACCCCCA CTATTATNNS NNSNNSNNST GGTATTTCGA CGTCTGGGG 49

(2) INFORMATION FOR SEQ ID NO:50:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 54 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

CACTATTATG GGAGCAGCCA CNNSNNSNNS NNSGTCTGGG GTCAAGGAAC 50

CCTG 54

(2) INFORMATION FOR SEQ ID NO:51:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 53 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

TCCTGTGCAG CTTCTGGCNN SNNSTTCNNS NNSNNSGGTA TGAACTGGGT 50

CCG 53

(2) INFORMATION FOR SEQ ID NO:52:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 52 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:

GAATGGGTTG GATGGATTAA CNNSNNSNNS GGTNNSCCGA CCTATGCTGC 50

GG 52

(2) INFORMATION FOR SEQ ID NO:53:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 49 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:53:

CTGTGCAAAG TACCCGNNST ATNNSNNSNN SNNSCACTGG TATTTCGAC 49

(2) INFORMATION FOR SEQ ID NO:54:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 48 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:54:

CGTTTCACTT TTTCTNNSGA CNNSTCCAAA NNSACAGCAT ACCTGCAG 48

(2) INFORMATION FOR SEQ ID NO:55:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 46 base pairs

(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:55:

GAATGGGTTG GATGGATTNN SNNSNNSNNS GGTGAACCGA CCTATG 46

(2) INFORMATION FOR SEQ ID NO:56:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:56:

Tyr Pro Tyr Tyr Arg Gly Thr Ser His Trp Tyr Phe Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:57:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:57:

Tyr Pro Tyr Tyr Ile Asn Lys Ser His Trp Tyr Phe Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:58:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:58:

Tyr Pro Tyr Tyr Tyr Gly Thr Ser His Trp Tyr Phe Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:59:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:59:

Tyr Pro Tyr Tyr Tyr Asn Gln Ser His Trp Tyr Phe Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:60:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:60:

Tyr Pro Tyr Tyr Ile Ala Lys Ser His Trp Tyr Phe Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:61:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:61:

Tyr Pro Tyr Tyr Arg Asp Asn Ser His Trp Tyr Phe Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:62:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:62:

Tyr Pro Tyr Tyr Trp Gly Thr Ser His Trp Tyr Phe Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:63:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:63:

Tyr Pro Tyr Tyr Arg Gln Asn Ser His Trp Tyr Phe Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:64:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:64:

Tyr Pro Tyr Tyr Arg Gln Ser Ser His Trp Tyr Phe Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:65:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:65:

Tyr Pro Tyr Tyr Arg Asn Thr Ser His Trp Tyr Phe Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:66:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:66:

Tyr Pro Tyr Tyr Lys Asn Thr Ser His Trp Tyr Phe Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:67:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:67:

Tyr Pro Tyr Tyr Ile Glu Arg Ser His Trp Tyr Phe Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:68:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:68:

Tyr Pro Tyr Tyr Arg Asn Ala Ser His Trp Tyr Phe Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:69:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:69:

Tyr Pro Tyr Tyr Thr Thr Arg Ser His Trp Tyr Phe Asp

1 5 10

(2) INFORMATION FOR SEQ ID NO:70:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:70:

Tyr Pro Tyr Tyr Glu Gly Ser Ser His Trp Tyr Phe Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:71:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:71:

Tyr Pro Tyr Tyr Arg Gln Arg Gly His Trp Tyr Phe Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:72:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:72:

Tyr Pro Tyr Tyr Thr Gly Arg Ser His Trp Tyr Phe Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:73:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:73:

Tyr Pro Tyr Tyr Thr Asn Thr Ser His Trp Tyr Phe Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:74:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:74:

Tyr Pro Tyr Tyr Arg Lys Gly Ser His Trp Tyr Phe Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:75:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:75:

Tyr Pro Tyr Tyr Thr Gly Ser Ser His Trp Tyr Phe Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:76:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:76:

Tyr Pro Tyr Tyr Arg Ser Gly Ser His Trp Tyr Phe Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:77:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:77:

Tyr Pro Tyr Tyr Thr Asn Arg Ser His Trp Tyr Phe Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:78:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:78:

Tyr Pro Tyr Tyr Arg Asn Ser Ser His Trp Tyr Phe Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:79:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:79:

Tyr Pro Tyr Tyr Lys Glu Ser Ser His Trp Tyr Phe Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:80:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:80:

Tyr Pro Tyr Tyr Arg Asp Ala Ser His Trp Tyr Phe Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:81:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:81:

Tyr Pro Tyr Tyr Arg Gln Lys Gly His Trp Tyr Phe Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:82:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:82:

Tyr Pro Tyr Tyr Lys Gly Gly Ser His Trp Tyr Phe Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:83:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:83:

Tyr Pro Tyr Tyr Tyr Gly Ala Ser His Trp Tyr Phe Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:84:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: Amino Acid

(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:84:

Tyr Pro Tyr Tyr Arg Gly Glu Ser His Trp Tyr Phe Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:85:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:85:

Tyr Pro Tyr Tyr Arg Ser Thr Ser His Trp Tyr Phe Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:86:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:86:

Gly Tyr Asp Phe Thr His Tyr Gly Met Asn
1 5 10

(2) INFORMATION FOR SEQ ID NO:87:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:87:

Gly Tyr Glu Phe Gln His Tyr Gly Met Asn
1 5 10

(2) INFORMATION FOR SEQ ID NO:88:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:88:

Gly Tyr Glu Phe Thr His Tyr Gly Met Asn
1 5 10

(2) INFORMATION FOR SEQ ID NO:89:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:89:

Gly Tyr Asp Phe Gly His Tyr Gly Met Asn
1 5 10

(2) INFORMATION FOR SEQ ID NO:90:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:90:

Gly Tyr Asp Phe Ser His Tyr Gly Met Asn
1 5 10

(2) INFORMATION FOR SEQ ID NO:91:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:91:

Gly Tyr Glu Phe Ser His Tyr Gly Met Asn
1 5 10

(2) INFORMATION FOR SEQ ID NO:92:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:92:

Phe Ser Val Asp Val Ser Lys Ser Thr Ala
1 5 10

(2) INFORMATION FOR SEQ ID NO:93:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:93:

Phe Ser Leu Asp Lys Ser Lys Ser Thr Ala
1 5 10

(2) INFORMATION FOR SEQ ID NO:94:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:94:

Phe Ser Leu Asp Val Trp Lys Ser Thr Ala
1 5 10

(2) INFORMATION FOR SEQ ID NO:95:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:95:

Phe Ser Ile Asp Lys Ser Lys Ser Thr Ala
1 5 10

(2) INFORMATION FOR SEQ ID NO:96:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:96:

GCAAAGTACC CGTACTATTA TGGGACGAGC CACTGGTATT TC 42

(2) INFORMATION FOR SEQ ID NO:97:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 48 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:97:

GTCACCATCA CCTGCAGCGC AAGTCAGGAT ATTAGCAACT ATTTAAAC 48

(2) INFORMATION FOR SEQ ID NO:98:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:98:

CCGTACTATT ATGGGAGCAG CCACTGGTAT TTC 33

(2) INFORMATION FOR SEQ ID NO:99:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6072 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:99:

GAATTCAACT TCTCCATACT TTGGATAAGG AAATACAGAC ATGAAAAATC 50

TCATTGCTGA GTTGTTATTT AAGCTTTGGA GATTATCGTC ACTGCAATGC 100

TTCGCAATAT GGCGCAAAAT GACCAACAGC GGTTGATTGA TCAGGTAGAG 150

GGGGCGCTGT ACGAGGTAAA GCCCGATGCC AGCATTCCTG ACGACGATAC 200

GGAGCTGCTG CGCGATTACG TAAAGAAGTT ATTGAAGCAT CCTCGTCAGT 250

AAAAAGTTAA TCTTTTCAAC AGCTGTCATA AAGTTGTCAC GGCCGAGACT 300

TATAGTCGCT TTGTTTTTAT TTTTTAATGT ATTTGTAACT AGAATTCGAG 350

CTCGGTACCC GGGGATCCTC TAGAGGTTGA GGTGATTTTA TGAAAAAGAA 400

TATCGCATTT CTTCTTGCAT CTATGTTCGT TTTTTCTATT GCTACAAACG 450

CGTACGCTGA TATCCAGTTG ACCCAGTCCC CGAGCTCCCT GTCCGCCTCT 500

GTGGGCGATA GGGTCACCAT CACCTGCAGC GCAAGTCAGG ATATTAGCAA 550

CTATTTAAAC TGGTATCAAC AGAAACCAGG AAAAGCTCCG AAACTACTGA 600

TTTACTTCAC CTCCTCTCTC CACTCTGGAG TCCCTTCTCG CTTCTCTGGA 650

TCCGGTTCTG GGACGGATTA CACTCTGACC ATCAGCAGTC TGCAGCCAGA 700

AGACTTCGCA ACTTATTACT GTCAACAGTA TAGCACCGTG CCGTGGACGT 750

TTGGACAGGG TACCAAGGTG GAGATCAAAC GAACTGTGGC TGCACCATCT 800

GTCTTCATCT TCCCGCCATC TGATGAGCAG TTGAAATCTG GAACTGCTTC 850

TGTTGTGTGC CTGCTGAATA ACTTCTATCC CAGAGAGGCC AAAGTACAGT 900

GGAAGGTGGA TAACGCCCTC CAATCGGGTA ACTCCCAGGA GAGTGTCACA 950

GAGCAGGACA GCAAGGACAG CACCTACAGC CTCAGCAGCA CCCTGACGCT 1000

GAGCAAAGCA GACTACGAGA AACACAAAGT CTACGCCTGC GAAGTCACCC 1050

ATCAGGGCCT GAGCTCGCCC GTCACAAAGA GCTTCAACAG GGGAGAGTGT 1100

TAAGCTGATC CTCTACGCCG GACGCATCGT GGCCCTAGTA CGCAACTAGT 1150

CGTAAAAAGG GTATCTAGAG GTTGAGGTGA TTTTATGAAA AAGAATATCG 1200

CATTTCTTCT TGCATCTATG TTCGTTTTTT CTATTGCTAC AAACGCGTAC 1250

GCTGAGGTTC AGCTGGTGGA GTCTGGCGGT GGCCTGGTGC AGCCAGGGGG 1300

CTCACTCCGT TTGTCCTGTG CAGCTTCTGG CTATACCTTC ACCAACTATG 1350

GTATGAACTG GATCCGTCAG GCCCCGGGTA AGGGCCTGGA ATGGGTTGGA 1400

TGGATTAACA CCTATACCGG TGAACCGACC TATGCTGCGG ATTTCAAACG 1450

TCGTTTTACT ATATCTGCAG ACACCTCCAG CAACACAGTT TACCTGCAGA 1500

TGAACAGCCT GCGCGCTGAG GACACTGCCG TCTATTACTG TGCAAAGTAC 1550

CCGCACTATT ATGGGAGCAG CCACTGGTAT TTCGACGTCT GGGGTCAAGG 1600

AACCCTGGTC ACCGTCTCCT CGGCCTCCAC CAAGGGCCCA TCGGTCTTCC 1650

CCCTGGCACC CTCCTCCAAG AGCACCTCTG GGGGCACAGC GGCCCTGGGC 1700

TGCCTGGTCA AGGACTACTT CCCCGAACCG GTGACGGTGT CGTGGAACTC 1750

AGGCGCCCTG ACCAGCGGCG TGCACACCTT CCCGGCTGTC CTACAGTCCT 1800

CAGGACTCTA CTCCCTCAGC AGCGTGGTGA CCGTGCCCTC CAGCAGCTTG 1850

GGCACCCAGA CCTACATCTG CAACGTGAAT CACAAGCCCA GCAACACCAA 1900

GGTCGACAAG AAAGTTGAGC CCAAATCTTG TGACAAAACT CACCTCTAGA 1950

GTGGCGGTGG CTCTGGTTCC GGTGATTTTG ATTATGAAAA GATGGCAAAC 2000

GCTAATAAGG GGGCTATGAC CGAAAATGCC GATGAAAACG CGCTACAGTC 2050

TGACGCTAAA GGCAAACTTG ATTCTGTCGC TACTGATTAC GGTGCTGCTA 2100

TCGATGGTTT CATTGGTGAC GTTTCCGGCC TTGCTAATGG TAATGGTGCT 2150

ACTGGTGATT TTGCTGGCTC TAATTCCCAA ATGGCTCAAG TCGGTGACGG 2200

TGATAATTCA CCTTTAATGA ATAATTTCCG TCAATATTTA CCTTCCCTCC 2250

CTCAATCGGT TGAATGTCGC CCTTTTGTCT TTAGCGCTGG TAAACCATAT 2300

GAATTTTCTA TTGATTGTGA CAAAATAAAC TTATTCCGTG GTGTCTTTGC 2350

GTTTCTTTTA TATGTTGCCA CCTTTATGTA TGTATTTTCT ACGTTTGCTA 2400

ACATACTGCG TAATAAGGAG TCTTAATCAT GCCAGTTCTT TTGGCTAGCG 2450

CCGCCCTATA CCTTGTCTGC CTCCCCGCGT TGCGTCGCGG TGCATGGAGC 2500

CGGGCCACCT CGACCTGAAT GGAAGCCGGC GGCACCTCGC TAACGGATTC 2550

ACCACTCCAA GAATTGGAGC CAATCAATTC TTGCGGAGAA CTGTGAATGC 2600

GCAAACCAAC CCTTGGCAGA ACATATCCAT CGCGTCCGCC ATCTCCAGCA 2650

GCCGCACGCG GCGCATCTCG GGCAGCGTTG GGTCCTGGCC ACGGGTGCGC 2700

ATGATCGTGC TCCTGTCGTT GAGGACCCGG CTAGGCTGGC GGGGTTGCCT 2750

TACTGGTTAG CAGAATGAAT CACCGATACG CGAGCGAACG TGAAGCGACT 2800

GCTGCTGCAA AACGTCTGCG ACCTGAGCAA CAACATGAAT GGTCTTCGGT 2850

TTCCGTGTTT CGTAAAGTCT GGAAACGCGG AAGTCAGCGC CCTGCACCAT 2900

TATGTTCCGG ATCTGCATCG CAGGATGCTG CTGGCTACCC TGTGGAACAC 2950

CTACATCTGT ATTAACGAAG CGCTGGCATT GACCCTGAGT GATTTTTCTC 3000

TGGTCCCGCC GCATCCATAC CGCCAGTTGT TTACCCTCAC AACGTTCCAG 3050

TAACCGGGCA TGTTCATCAT CAGTAACCCG TATCGTGAGC ATCCTCTCTC 3100

GTTTCATCGG TATCATTACC CCCATGAACA GAAATTCCCC CTTACACGGA 3150

GGCATCAAGT GACCAAACAG GAAAAAACCG CCCTTAACAT GGCCCGCTTT 3200

ATCAGAAGCC AGACATTAAC GCTTCTGGAG AAACTCAACG AGCTGGACGC 3250

GGATGAACAG GCAGACATCT GTGAATCGCT TCACGACCAC GCTGATGAGC 3300

TTTACCGCAG GATCCGGAAA TTGTAAACGT TAATATTTTG TTAAAATTCG 3350

CGTTAAATTT TTGTTAAATC AGCTCATTTT TTAACCAATA GGCCGAAATC 3400

GGCAAAATCC CTTATAAATC AAAAGAATAG ACCGAGATAG GGTTGAGTGT 3450

TGTTCCAGTT TGGAACAAGA GTCCACTATT AAAGAACGTG GACTCCAACG 3500

TCAAAGGGCG AAAAACCGTC TATCAGGGCT ATGGCCCACT ACGTGAACCA 3550

TCACCCTAAT CAAGTTTTTT GGGGTCGAGG TGCCGTAAAG CACTAAATCG 3600

GAACCCTAAA GGGAGCCCCC GATTTAGAGC TTGACGGGGA AAGCCGGCGA 3650

ACGTGGCGAG AAAGGAAGGG AAGAAAGCGA AAGGAGCGGG CGCTAGGGCG 3700

CTGGCAAGTG TAGCGGTCAC GCTGCGCGTA ACCACCACAC CCGCCGCGCT 3750

TAATGCGCCG CTACAGGGCG CGTCCGGATC CTGCCTCGCG CGTTTCGGTG 3800

ATGACGGTGA AAACCTCTGA CACATGCAGC TCCCGGAGAC GGTCACAGCT 3850

TGTCTGTAAG CGGATGCCGG GAGCAGACAA GCCCGTCAGG GCGCGTCAGC 3900

GGGTGTTGGC GGGTGTCGGG GCGCAGCCAT GACCCAGTCA CGTAGCGATA 3950

GCGGAGTGTA TACTGGCTTA ACTATGCGGC ATCAGAGCAG ATTGTACTGA 4000

GAGTGCACCA TATGCGGTGT GAAATACCGC ACAGATGCGT AAGGAGAAAA 4050

TACCGCATCA GGCGCTCTTC CGCTTCCTCG CTCACTGACT CGCTGCGCTC 4100

GGTCGTTCGG CTGCGGCGAG CGGTATCAGC TCACTCAAAG GCGGTAATAC 4150

GGTTATCCAC AGAATCAGGG GATAACGCAG GAAAGAACAT GTGAGCAAAA 4200

GGCCAGCAAA AGGCCAGGAA CCGTAAAAAG GCCGCGTTGC TGGCGTTTTT 4250

CCATAGGCTC CGCCCCCCTG ACGAGCATCA CAAAAATCGA CGCTCAAGTC 4300

AGAGGTGGCG AAACCCGACA GGACTATAAA GATACCAGGC GTTTCCCCCT 4350

GGAAGCTCCC TCGTGCGCTC TCCTGTTCCG ACCCTGCCGC TTACCGGATA 4400

CCTGTCCGCC TTTCTCCCTT CGGGAAGCGT GGCGCTTTCT CATAGCTCAC 4450

GCTGTAGGTA TCTCAGTTCG GTGTAGGTCG TTCGCTCCAA GCTGGGCTGT 4500

GTGCACGAAC CCCCCGTTCA GCCCGACCGC TGCGCCTTAT CCGGTAACTA 4550

TCGTCTTGAG TCCAACCCGG TAAGACACGA CTTATCGCCA CTGGCAGCAG 4600

CCACTGGTAA CAGGATTAGC AGAGCGAGGT ATGTAGGCGG TGCTACAGAG 4650

TTCTTGAAGT GGTGGCCTAA CTACGGCTAC ACTAGAAGGA CAGTATTTGG 4700

TATCTGCGCT CTGCTGAAGC CAGTTACCTT CGGAAAAAGA GTTGGTAGCT 4750

CTTGATCCGG CAAACAAACC ACCGCTGGTA GCGGTGGTTT TTTTGTTTGC 4800

AAGCAGCAGA TTACGCGCAG AAAAAAAGGA TCTCAAGAAG ATCCTTTGAT 4850

CTTTTCTACG GGGTCTGACG CTCAGTGGAA CGAAAACTCA CGTTAAGGGA 4900

TTTTGGTCAT GAGATTATCA AAAAGGATCT TCACCTAGAT CCTTTTAAAT 4950

TAAAAATGAA GTTTTAAATC AATCTAAAGT ATATATGAGT AAACTTGGTC 5000

TGACAGTTAC CAATGCTTAA TCAGTGAGGC ACCTATCTCA GCGATCTGTC 5050

TATTTCGTTC ATCCATAGTT GCCTGACTCC CCGTCGTGTA GATAACTACG 5100

ATACGGGAGG GCTTACCATC TGGCCCCAGT GCTGCAATGA TACCGCGAGA 5150

CCCACGCTCA CCGGCTCCAG ATTTATCAGC AATAAACCAG CCAGCCGGAA 5200

GGGCCGAGCG CAGAAGTGGT CCTGCAACTT TATCCGCCTC CATCCAGTCT 5250

ATTAATTGTT GCCGGGAAGC TAGAGTAAGT AGTTCGCCAG TTAATAGTTT 5300

GCGCAACGTT GTTGCCATTG CTGCAGGCAT CGTGGTGTCA CGCTCGTCGT 5350

TTGGTATGGC TTCATTCAGC TCCGGTTCCC AACGATCAAG GCGAGTTACA 5400

TGATCCCCCA TGTTGTGCAA AAAAGCGGTT AGCTCCTTCG GTCCTCCGAT 5450

CGTTGTCAGA AGTAAGTTGG CCGCAGTGTT ATCACTCATG GTTATGGCAG 5500

CACTGCATAA TTCTCTTACT GTCATGCCAT CCGTAAGATG CTTTTCTGTG 5550

ACTGGTGAGT ACTCAACCAA GTCATTCTGA GAATAGTGTA TGCGGCGACC 5600

GAGTTGCTCT TGCCCGGCGT CAACACGGGA TAATACCGCG CCACATAGCA 5650

GAACTTTAAA AGTGCTCATC ATTGGAAAAC GTTCTTCGGG GCGAAAACTC 5700

TCAAGGATCT TACCGCTGTT GAGATCCAGT TCGATGTAAC CCACTCGTGC 5750

ACCCAACTGA TCTTCAGCAT CTTTTACTTT CACCAGCGTT TCTGGGTGAG 5800

CAAAAACAGG AAGGCAAAAT GCCGCAAAAA AGGGAATAAG GGCGACACGG 5850

AAATGTTGAA TACTCATACT CTTCCTTTTT CAATATTATT GAAGCATTTA 5900

TCAGGGTTAT TGTCTCATGA GCGGATACAT ATTTGAATGT ATTTAGAAAA 5950

ATAAACAAAT AGGGGTTCCG CGCACATTTC CCCGAAAAGT GCCACCTGAC 6000

GTCTAAGAAA CCATTATTAT CATGACATTA ACCTATAAAA ATAGGCGTAT 6050

CACGAGGCCC TTTCGTCTTC AA 6072

(2) INFORMATION FOR SEQ ID NO:100:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 237 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:100:

```
Met Lys Lys Asn Ile Ala Phe Leu Leu Ala Ser Met Phe Val Phe
-23         -20                 -15                 -10

Ser Ile Ala Thr Asn Ala Tyr Ala Asp Ile Gln Leu Thr Gln Ser
             -5                   1                   5

Pro Ser Ser Leu Ser Ala Ser Val Gly Asp Arg Val Thr Ile Thr
         10                  15                  20

Cys Ser Ala Ser Gln Asp Ile Ser Asn Tyr Leu Asn Trp Tyr Gln
         25                  30                  35

Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile Tyr Phe Thr Ser
         40                  45                  50

Ser Leu His Ser Gly Val Pro Ser Arg Phe Ser Gly Ser Gly Ser
         55                  60                  65

Gly Thr Asp Tyr Thr Leu Thr Ile Ser Ser Leu Gln Pro Glu Asp
         70                  75                  80

Phe Ala Thr Tyr Tyr Cys Gln Gln Tyr Ser Thr Val Pro Trp Thr
         85                  90                  95
```

```
Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr Val Ala Ala
        100                 105                 110

Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser
        115                 120                 125

Gly Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg
        130                 135                 140

Glu Ala Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly
        145                 150                 155

Asn Ser Gln Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr
        160                 165                 170

Tyr Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu
        175                 180                 185

Lys His Lys Val Tyr Ala Cys Glu Val Thr His Gln Gly Leu Ser
        190                 195                 200

Ser Pro Val Thr Lys Ser Phe Asn Arg Gly Glu Cys
        205                 210
```

(2) INFORMATION FOR SEQ ID NO:101:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 254 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:101:

```
Met Lys Lys Asn Ile Ala Phe Leu Leu Ala Ser Met Phe Val Phe
-23         -20                 -15                 -10

Ser Ile Ala Thr Asn Ala Tyr Ala Glu Val Gln Leu Val Glu Ser
             -5                   1                   5

Gly Gly Gly Leu Val Gln Pro Gly Gly Ser Leu Arg Leu Ser Cys
         10                  15                  20

Ala Ala Ser Gly Tyr Thr Phe Thr Asn Tyr Gly Met Asn Trp Ile
         25                  30                  35

Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val Gly Trp Ile Asn
         40                  45                  50

Thr Tyr Thr Gly Glu Pro Thr Tyr Ala Ala Asp Phe Lys Arg Arg
         55                  60                  65

Phe Thr Ile Ser Ala Asp Thr Ser Ser Asn Thr Val Tyr Leu Gln
         70                  75                  80

Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys Ala
         85                  90                  95
```

```
Lys Tyr Pro His Tyr Tyr Gly Ser Ser His Trp Tyr Phe Asp Val
        100                 105                 110

Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys
        115                 120                 125

Gly Pro Ser Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser
        130                 135                 140

Gly Gly Thr Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro
        145                 150                 155

Glu Pro Val Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly
        160                 165                 170

Val His Thr Phe Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser
        175                 180                 185

Leu Ser Ser Val Val Thr Val Pro Ser Ser Ser Leu Gly Thr Gln
        190                 195                 200

Thr Tyr Ile Cys Asn Val Asn His Lys Pro Ser Asn Thr Lys Val
        205                 210                 215

Asp Lys Lys Val Glu Pro Lys Ser Cys Asp Lys Thr His Leu
        220                 225                 230
```

(2) INFORMATION FOR SEQ ID NO:102:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 158 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:102:

```
Ser Gly Gly Gly Ser Gly Ser Gly Asp Phe Asp Tyr Glu Lys Met
  1               5                  10                  15

Ala Asn Ala Asn Lys Gly Ala Met Thr Glu Asn Ala Asp Glu Asn
                20                  25                  30

Ala Leu Gln Ser Asp Ala Lys Gly Lys Leu Asp Ser Val Ala Thr
                35                  40                  45

Asp Tyr Gly Ala Ala Ile Asp Gly Phe Ile Gly Asp Val Ser Gly
                50                  55                  60

Leu Ala Asn Gly Asn Gly Ala Thr Gly Asp Phe Ala Gly Ser Asn
                65                  70                  75

Ser Gln Met Ala Gln Val Gly Asp Gly Asp Asn Ser Pro Leu Met
                80                  85                  90

Asn Asn Phe Arg Gln Tyr Leu Pro Ser Leu Pro Gln Ser Val Glu
                95                 100                 105
```

Cys Arg Pro Phe Val Phe Ser Ala Gly Lys Pro Tyr Glu Phe Ser
110 115 120

Ile Asp Cys Asp Lys Ile Asn Leu Phe Arg Gly Val Phe Ala Phe
125 130 135

Leu Leu Tyr Val Ala Thr Phe Met Tyr Val Phe Ser Thr Phe Ala
140 145 150

Asn Ile Leu Arg Asn Lys Glu Ser
155

(2) INFORMATION FOR SEQ ID NO:103:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 110 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:103:

Asp Ile Gln Leu Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
1 5 10 15

Gly Asp Arg Val Thr Ile Thr Cys Ser Ala Ser Gln Asp Ile Ser
20 25 30

Asn Tyr Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
35 40 45

Leu Leu Ile Tyr Phe Thr Ser Ser Leu His Ser Gly Val Pro Ser
50 55 60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Tyr Thr Leu Thr Ile
65 70 75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
80 85 90

Tyr Ser Thr Val Pro Trp Thr Phe Gly Gln Gly Thr Lys Val Glu
95 100 105

Ile Lys Arg Thr Val
110

(2) INFORMATION FOR SEQ ID NO:104:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 118 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:104:

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
1 5 10 15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr
20 25 30

Asn Tyr Gly Met Asn Trp Ile Arg Gln Ala Pro Gly Lys Gly Leu
35 40 45

Glu Trp Val Gly Trp Ile Asn Thr Tyr Thr Gly Glu Pro Thr Tyr
50 55 60

Ala Ala Asp Phe Lys Arg Arg Phe Thr Ile Ser Ala Asp Thr Ser
65 70 75

Ser Asn Thr Val Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
80 85 90

Thr Ala Val Tyr Tyr Cys Ala Lys Tyr Pro His Tyr Tyr Gly Ser
95 100 105

Ser His Trp Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu
110 115

(2) INFORMATION FOR SEQ ID NO:105:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 110 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:105:

Asp Ile Gln Leu Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
1 5 10 15

Gly Asp Arg Val Thr Ile Thr Cys Ser Ala Ser Gln Asp Ile Ser
20 25 30

Asn Tyr Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
35 40 45

Val Leu Ile Tyr Phe Thr Ser Ser Leu His Ser Gly Val Pro Ser
50 55 60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Tyr Thr Leu Thr Ile
65 70 75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
80 85 90

Tyr Ser Thr Val Pro Trp Thr Phe Gly Gln Gly Thr Lys Val Glu
95 100 105

Ile Lys Arg Thr Val
110

(2) INFORMATION FOR SEQ ID NO:106:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 118 amino acids

(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:106:

```
Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
 1               5                  10                  15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr
            20                  25                  30

Asn Tyr Gly Met Asn Trp Ile Arg Gln Ala Pro Gly Lys Gly Leu
            35                  40                  45

Glu Trp Val Gly Trp Ile Asn Thr Tyr Thr Gly Glu Pro Thr Tyr
            50                  55                  60

Ala Ala Asp Phe Lys Arg Arg Phe Thr Phe Ser Ala Asp Thr Ser
            65                  70                  75

Ser Asn Thr Val Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
            80                  85                  90

Thr Ala Val Tyr Tyr Cys Ala Lys Tyr Pro His Tyr Tyr Gly Ser
            95                 100                 105

Ser His Trp Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu
           110                 115
```

(2) INFORMATION FOR SEQ ID NO:107:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 110 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:107:

```
Asp Ile Gln Leu Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
 1               5                  10                  15

Gly Asp Arg Val Thr Ile Thr Cys Ser Ala Ser Gln Asp Ile Ser
            20                  25                  30

Asn Tyr Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
            35                  40                  45

Val Leu Ile Tyr Phe Thr Ser Ser Leu His Ser Gly Val Pro Ser
            50                  55                  60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
            65                  70                  75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
            80                  85                  90

Tyr Ser Thr Val Pro Trp Thr Phe Gly Gln Gly Thr Lys Val Glu
            95                 100                 105
```

Ile Lys Arg Thr Val
110

(2) INFORMATION FOR SEQ ID NO:108:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 118 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:108:

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
1 5 10 15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr
20 25 30

Asn Tyr Gly Met Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
35 40 45

Glu Trp Val Gly Trp Ile Asn Thr Tyr Thr Gly Glu Pro Thr Tyr
50 55 60

Ala Ala Asp Phe Lys Arg Arg Phe Thr Phe Ser Leu Asp Thr Ser
65 70 75

Lys Ser Thr Ala Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
80 85 90

Thr Ala Val Tyr Tyr Cys Ala Lys Tyr Pro His Tyr Tyr Gly Ser
95 100 105

Ser His Trp Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu
110 115

(2) INFORMATION FOR SEQ ID NO:109:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 110 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:109:

Asp Ile Gln Leu Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
1 5 10 15

Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Asn Glu Gln Leu Ser
20 25 30

Asn Tyr Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
35 40 45

Val Leu Ile Tyr Phe Thr Ser Ser Leu His Ser Gly Val Pro Ser
50 55 60

```
Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
                65                  70                  75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
                80                  85                  90

Tyr Ser Thr Val Pro Trp Thr Phe Gly Gln Gly Thr Lys Val Glu
                95                 100                 105

Ile Lys Arg Thr Val
               110
```

(2) INFORMATION FOR SEQ ID NO:110:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 118 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:110:

```
Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
  1               5                  10                  15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr
                20                  25                  30

Asn Tyr Gly Ile Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
                35                  40                  45

Glu Trp Val Gly Trp Ile Asn Thr Tyr Thr Gly Glu Pro Thr Tyr
                50                  55                  60

Ala Ala Asp Phe Lys Arg Arg Phe Thr Phe Ser Leu Asp Thr Ser
                65                  70                  75

Lys Ser Thr Ala Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
                80                  85                  90

Thr Ala Val Tyr Tyr Cys Ala Lys Tyr Pro His Tyr Tyr Gly Ser
                95                 100                 105

Ser His Trp Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu
               110                 115
```

(2) INFORMATION FOR SEQ ID NO:111:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 110 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:111:

```
Asp Ile Gln Leu Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
  1               5                  10                  15
```

```
Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Asn Glu Gln Leu Ser
                  20                  25                  30

Asn Tyr Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
                  35                  40                  45

Val Leu Ile Tyr Phe Thr Ser Ser Leu His Ser Gly Val Pro Ser
                  50                  55                  60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
                  65                  70                  75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
                  80                  85                  90

Tyr Ser Thr Val Pro Trp Thr Phe Gly Gln Gly Thr Lys Val Glu
                  95                 100                 105

Ile Lys Arg Thr Val
                 110
```

(2) INFORMATION FOR SEQ ID NO:112:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 118 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:112:

```
Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
  1               5                  10                  15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Asp Phe Thr
                  20                  25                  30

His Tyr Gly Met Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
                  35                  40                  45

Glu Trp Val Gly Trp Ile Asn Thr Tyr Thr Gly Glu Pro Thr Tyr
                  50                  55                  60

Ala Ala Asp Phe Lys Arg Arg Phe Thr Phe Ser Leu Asp Thr Ser
                  65                  70                  75

Lys Ser Thr Ala Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
                  80                  85                  90

Thr Ala Val Tyr Tyr Cys Ala Lys Tyr Pro His Tyr Tyr Gly Ser
                  95                 100                 105

Ser His Trp Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu
                 110                 115
```

(2) INFORMATION FOR SEQ ID NO:113:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 110 amino acids

(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:113:

```
Asp Ile Gln Leu Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
 1               5                  10                  15

Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Asn Glu Gln Leu Ser
            20                  25                  30

Asn Tyr Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
            35                  40                  45

Val Leu Ile Tyr Phe Thr Ser Ser Leu His Ser Gly Val Pro Ser
            50                  55                  60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
            65                  70                  75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
            80                  85                  90

Tyr Ser Thr Val Pro Trp Thr Phe Gly Gln Gly Thr Lys Val Glu
            95                 100                 105

Ile Lys Arg Thr Val
           110
```

(2) INFORMATION FOR SEQ ID NO:114:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 118 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:114:

```
Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
 1               5                  10                  15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr
            20                  25                  30

Asn Tyr Gly Ile Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
            35                  40                  45

Glu Trp Val Gly Trp Ile Asn Thr Tyr Thr Gly Glu Pro Thr Tyr
            50                  55                  60

Ala Ala Asp Phe Lys Arg Arg Phe Thr Phe Ser Leu Asp Thr Ser
            65                  70                  75

Lys Ser Thr Ala Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
            80                  85                  90

Thr Ala Val Tyr Tyr Cys Ala Lys Tyr Pro Tyr Tyr Tyr Gly Thr
            95                 100                 105
```

Ser His Trp Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu
110 115

(2) INFORMATION FOR SEQ ID NO:115:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 110 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:115:

Asp Ile Gln Leu Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
1 5 10 15

Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Asn Glu Gln Leu Ser
20 25 30

Asn Tyr Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
35 40 45

Val Leu Ile Tyr Phe Thr Ser Ser Leu His Ser Gly Val Pro Ser
50 55 60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
65 70 75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
80 85 90

Tyr Ser Thr Val Pro Trp Thr Phe Gly Gln Gly Thr Lys Val Glu
95 100 105

Ile Lys Arg Thr Val
110

(2) INFORMATION FOR SEQ ID NO:116:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 118 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:116:

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
1 5 10 15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Asp Phe Thr
20 25 30

His Tyr Gly Met Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
35 40 45

Glu Trp Val Gly Trp Ile Asn Thr Tyr Thr Gly Glu Pro Thr Tyr
50 55 60

```
Ala Ala Asp Phe Lys Arg Arg Phe Thr Phe Ser Leu Asp Thr Ser
                  65                  70                  75

Lys Ser Thr Ala Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
                  80                  85                  90

Thr Ala Val Tyr Tyr Cys Ala Lys Tyr Pro Tyr Tyr Tyr Gly Thr
                  95                 100                 105

Ser His Trp Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu
                 110                 115
```

(2) INFORMATION FOR SEQ ID NO:117:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 110 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:117:

```
Asp Ile Gln Leu Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
 1               5                  10                  15

Gly Asp Arg Val Thr Ile Thr Cys Ser Ala Ser Gln Asp Ile Ser
                 20                  25                  30

Asn Tyr Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
                 35                  40                  45

Val Leu Ile Tyr Phe Thr Ser Ser Leu His Ser Gly Val Pro Ser
                 50                  55                  60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
                 65                  70                  75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
                 80                  85                  90

Tyr Ser Thr Val Pro Trp Thr Phe Gly Gln Gly Thr Lys Val Glu
                 95                 100                 105

Ile Lys Arg Thr Val
                110
```

(2) INFORMATION FOR SEQ ID NO:118:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 118 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:118:

```
Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
 1               5                  10                  15
```

```
Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Asp Phe Thr
                  20                  25                  30

His Tyr Gly Met Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
                  35                  40                  45

Glu Trp Val Gly Trp Ile Asn Thr Tyr Thr Gly Glu Pro Thr Tyr
                  50                  55                  60

Ala Ala Asp Phe Lys Arg Arg Phe Thr Phe Ser Leu Asp Thr Ser
                  65                  70                  75

Lys Ser Thr Ala Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
                  80                  85                  90

Thr Ala Val Tyr Tyr Cys Ala Lys Tyr Pro Tyr Tyr Tyr Gly Thr
                  95                 100                 105

Ser His Trp Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu
                 110                 115
```

(2) INFORMATION FOR SEQ ID NO:119:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:119:

```
Gly Tyr Xaa Xaa Xaa Xaa Tyr Gly Xaa Asn
 1               5                  10
```

(2) INFORMATION FOR SEQ ID NO:120:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:120:

```
Trp Ile Asn Thr Xaa Thr Gly Glu Pro Thr Tyr Ala Ala Asp Phe
 1               5                  10                  15

Lys Arg
```

(2) INFORMATION FOR SEQ ID NO:121:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:121:

Tyr Pro Xaa Tyr Xaa Xaa Xaa Xaa His Trp Tyr Phe Asp Val
1 5 10

(2) INFORMATION FOR SEQ ID NO:122:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:122:

Xaa Ser Xaa Asp Xaa Xaa Xaa Xaa Thr Xaa
1 5 10

(2) INFORMATION FOR SEQ ID NO:123:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:123:

Xaa Ala Xaa Xaa Xaa Xaa Ser Asn Tyr Leu Asn
1 5 10

(2) INFORMATION FOR SEQ ID NO:124:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:124:

Phe Thr Ser Ser Leu His Ser
1 5

(2) INFORMATION FOR SEQ ID NO:125:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:125:

Gln Gln Tyr Ser Xaa Xaa Pro Trp Thr
1 5

(2) INFORMATION FOR SEQ ID NO:126:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 108 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:126:

```
Asp Ile Gln Xaa Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
 1               5                  10                  15

Gly Asp Arg Val Thr Ile Thr Cys Ser Ala Ser Gln Asp Ile Ser
            20                  25                  30

Asn Tyr Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
            35                  40                  45

Val Leu Ile Tyr Phe Thr Ser Ser Leu His Ser Gly Val Pro Ser
            50                  55                  60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
            65                  70                  75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
            80                  85                  90

Tyr Ser Thr Val Pro Trp Thr Phe Gly Gln Gly Thr Lys Val Glu
            95                  100                 105

Ile Lys Arg
```

(2) INFORMATION FOR SEQ ID NO:127:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 123 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:127:

```
Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
 1               5                  10                  15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Xaa Phe Thr
            20                  25                  30

Xaa Tyr Gly Met Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
            35                  40                  45

Glu Trp Val Gly Trp Ile Asn Thr Tyr Thr Gly Glu Pro Thr Tyr
            50                  55                  60

Ala Ala Asp Phe Lys Arg Arg Phe Thr Phe Ser Leu Asp Thr Ser
            65                  70                  75

Lys Ser Thr Ala Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
            80                  85                  90

Thr Ala Val Tyr Tyr Cys Ala Lys Tyr Pro Xaa Tyr Tyr Gly Xaa
            95                  100                 105

Ser His Trp Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu Val Thr
           110                 115                 120
```

Val Ser Ser

(2) INFORMATION FOR SEQ ID NO:128:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:128:

Gly Tyr Asp Phe Thr His Tyr Gly Met Asn
1 5 10

(2) INFORMATION FOR SEQ ID NO:129:

Ins C4

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:129:

Tyr Pro Tyr Tyr Tyr Gly Thr Ser His Trp Tyr Phe Asp Val
1 5 10

(2) INFORMATION FOR SEQ ID NO:130:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:130:

Gly Tyr Xaa Phe Thr Xaa Tyr Gly Met Asn
1 5 10

(2) INFORMATION FOR SEQ ID NO:131:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:131:

Tyr Pro Xaa Tyr Tyr Gly Xaa Ser His Trp Tyr Phe Asp Val
1 5 10

UNITED STATES PATENT AND TRADEMARK OFFICE

COMMISSIONER FOR PATENTS
UNITED STATES PATENT AND TRADEMARK OFFICE
WASHINGTON, D.C. 20231
www.uspto.gov

APPLICATION NUMBER	FILING/RECEIPT DATE	FIRST NAMED APPLICANT	ATTORNEY DOCKET NUMBER
09/723,752	11/27/2000	Manuel Baca	P1093P1D1

CONFIRMATION NO. 6340

FORMALITIES LETTER

OC000000007228019

Attn: Steven X. Cui
GENENTECH, INC.
1 DNA Way
South San Francisco, CA 94080-4990

Date Mailed: 12/26/2001

NOTICE TO COMPLY WITH REQUIREMENTS FOR PATENT APPLICATIONS CONTAINING NUCLEOTIDE SEQUENCE AND/OR AMINO ACID SEQUENCE DISCLOSURES

Applicant is given **TWO MONTHS FROM THE DATE OF THIS NOTICE** within which to file the items indicated below to avoid abandonment. Extensions of time may be obtained under the provisions of 37 CFR 1.136(a).

- This application does not contain, as a separate part of the disclosure on paper copy, a "Sequence Listing" as required by 37 C.F.R. 1.821(c) Applicant must provide an initial paper or compact disc copy of the "Sequence Listing", as well as an amendment directing its entry into the application and a statement that the content of the sequence listing information recorded in computer readable form is identical to the written (on paper or compact disc) sequence listing and, where applicable, includes no new matter, as required by 37 CFR 1.821(e), 1.821(f), 1.821(g), 1.825(b), or 1.825(d). If the effective filing date is on or after September 8, 2000, see the final rulemaking notice published in the Federal Register at 65 FR 54604 (September 8, 2000) and 1238 OG 145 (September 19, 2000).

For questions regarding compliance to these requirements, please contact:

- **For Rules Interpretation, call (703) 308-4216**
- **To Purchase PatentIn Software, call (703) 306-2600**
- **For PatentIn Software Program Help, call (703) 306-4119 or e-mail at patin21help@uspto.gov or patin3help@uspto.gov**

A copy of this notice MUST be returned with the reply.

Customer Service Center
Initial Patent Examination Division (703) 308-1202
PART 2 - COPY TO BE RETURNED WITH RESPONSE

m

0360

Patent Docket P1093P1D1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Manuel Baca et al. Serial No.: 09/723,752 Filed: November 27, 2000 For: ANTI-VEGF ANTIBODIES	Group Art Unit: 1614 Examiner: Not yet assigned CONFIRMATION NO: CERTIFICATE OF MAILING I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Assistant Commissioner of Patents, Washington, D.C. 20231 on February 12, 2002 Mona Beltran

RESPONSE TO NOTICE TO COMPLY WITH REQUIREMENTS FOR PATENT APPLICATIONS CONTAINING NUCLEOTIDE SEQUENCE AND/OR AMINO ACID SEQUENCE DISCLOSURES

Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

This is responsive to the Notice to Comply with Requirements for Patent Applications Containing Nucleotide Sequence and/or Amino Acid Sequence Disclosures - Filing Date Granted dated December 26, 2001. Transmitted herewith are the following documents:

1. A paper copy of the Sequence Listing for USSN 08/908,469.
2. Certificate Re: Sequence Listing.
3. Copy of Letter and Request To Use Computer-Readable Sequence Listing Under 37 CFR §1.821(e) that was mailed to USPTO on June 26, 2001.
4. Copy of Notice to Comply with Requirements for Patent Applications Containing Nucleotide Sequence and/or Amino Acid Sequence Disclosures

Respectfully submitted,

GENENTECH, INC.

Date: February 12, 2002

By: _____
Steven X. Cui
Reg. No. 44,637
Telephone No. (650) 225-8674

09157
PATENT TRADEMARK OFFICE

#103668

Patent Docket P1093P1D1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of Manuel Baca et al. Serial No.: 09/723,752 Filed: November 27, 2000 For: ANTI-VEGF ANTIBODIES	Group Art Unit: 1614 Examiner: Not yet assigned CERTIFICATE OF MAILING I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Assistant Commissioner of Patents, Washington, D.C. 20231 on February 12, 2002 Mona Beltran

CERTIFICATE RE: SEQUENCE LISTING

RESPONSE UNDER 37 CFR § 1.821(f) and (g)

Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

I hereby state that the Sequence Listing submitted herewith is submitted in paper copy. The information recorded in computer readable form (as submitted in application Serial No. 08/908,469, filed August 6, 1997,) is identical to the written sequence listing. I further state that this submission includes no new matter.

Respectfully submitted,

GENENTECH, INC.

Date: February 12, 2002

By: _____
Steven X. Cui
Reg. No. 44,637
Telephone No. (650) 225-8674

09157
PATENT TRADEMARK OFFICE

#103675

Patent Docket P1093P1D1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Manuel Baca et al. Serial No.: 09/723,752 Filed: November 27, 2000 For: ANTI-VEGF ANTIBODIES	Group Art Unit: 1647 Examiner: CERTIFICATE OF MAILING I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Assistant Commissioner of Patents, Washington, D.C. 20231 on June 26, 2001 Eileen Ly

Letter and REQUEST TO USE COMPUTER-READABLE SEQUENCE LISTING

UNDER 37 CFR §1.821(e)

Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

Applicants respectfully request that the compliant computer-readable Sequence Listing filed in application Serial No. 08/908,469 be used as the computer-readable Sequence Listing for the present, above-identified application.

The paper copy of the Sequence Listing filed in the present application is identical to the computer-readable copy of the Sequence Listing filed in the application Serial No. 08/908,469.

Respectfully submitted,

GENENTECH, INC.

Date: June 26 2001

By: Steven X. Cui

Steven X. Cui
Reg. No. 44,637
Telephone No. (650) 225-8674

09157
PATENT TRADEMARK OFFICE

#91819

COPY

UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office

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OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

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CHANGE OF ADDRESS/POWER OF ATTORNEY

FILE LOCATION 16C1 SERIAL NUMBER 09723752 PATENT NUMBER

THE CORRESPONDENCE ADDRESS HAS BEEN CHANGED TO CUSTOMER # 9157

THE PRACTITIONERS OF RECORD HAVE BEEN CHANGED TO CUSTOMER # 9157

THE FEE ADDRESS HAS BEEN CHANGED TO CUSTOMER # 9157

ON 06/25/02 THE ADDRESS OF RECORD FOR CUSTOMER NUMBER 9157 IS:

GENENTECH, INC.
1 DNA WAY
SOUTH SAN FRANCISCO CA 94080

AND THE PRACTITIONERS OF RECORD FOR CUSTOMER NUMBER 9157 ARE:

28616	32037	32171	35059	35600	35910	36487	36575	39044	39447
40378	40887	42767	44637	45945	49075				

PTO INSTRUCTIONS: PLEASE TAKE THE FOLLOWING ACTION WHEN THE CORRESPONDENCE ADDRESS HAS BEEN CHANGED TO CUSTOMER NUMBER: RECORD, ON THE NEXT AVAILABLE CONTENTS LINE OF THE FILE JACKET, 'ADDRESS CHANGE TO CUSTOMER NUMBER'. LINE THROUGH THE OLD ADDRESS ON THE FILE JACKET LABEL AND ENTER ONLY THE 'CUSTOMER NUMBER' AS THE NEW ADDRESS. FILE THIS LETTER IN THE FILE JACKET. WHEN ABOVE CHANGES ARE ONLY TO FEE ADDRESS AND/OR PRACTITIONERS OF RECORD, FILE LETTER IN THE FILE JACKET.
THIS FILE IS ASSIGNED TO GAU 1614.

PTO-FMD
TALBOT-1/97

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
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Washington, D.C. 20231
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/723,752	11/27/2000	Manuel Baca	P1093P1D1	6340

9157 7590 08/28/2002

GENENTECH, INC.
1 DNA WAY
SOUTH SAN FRANCISCO, CA 94080

EXAMINER
HELMS, LARRY RONALD

ART UNIT	PAPER NUMBER
1642	11

DATE MAILED: 08/28/2002

Please find below and/or attached an Office communication concerning this application or proceeding.

PTO-90C (Rev. 07-01)

Offic Action Summary	Application No. 09/723,752	Applicant(s) BACA ET AL.	
	Examiner Larry R. Helms	Art Unit 1642	

-- Th MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 1 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133).
- Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☐ Responsive to communication(s) filed on _____ .

2a)☐ This action is **FINAL**. 2b)☐ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) 43-59 is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☐ Claim(s) _____ is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☒ Claim(s) 43-59 are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

11)☐ The proposed drawing correction filed on _____ is: a)☐ approved b)☐ disapproved by the Examiner.

If approved, corrected drawings are required in reply to this Office action.

12)☐ The oath or declaration is objected to by the Examiner.

Priority under 35 U.S.C. §§ 119 and 120

13)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____ .

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

14)☐ Acknowledgment is made of a claim for domestic priority under 35 U.S.C. § 119(e) (to a provisional application).

a) ☐ The translation of the foreign language provisional application has been received.

15)☐ Acknowledgment is made of a claim for domestic priority under 35 U.S.C. §§ 120 and/or 121.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO-1449) Paper No(s) _____ .
4) ☐ Interview Summary (PTO-413) Paper No(s). _____ .
5) ☐ Notice of Informal Patent Application (PTO-152)
6) ☐ Other: .

DETAILED ACTION

Election/Restrictions

1. This application contains claims directed to the following patentably distinct species of the claimed invention: claims 47-52 are directed to specific SEQ ID Nos for CDRs and for entire antibodies. Applicant is required to pick either three CDRs from the light chain and a heavy chain or an entire light chain and heavy chain.

The sequences are distinct because each is a specific sequence and art on one sequence would not necessarily be art on another.

Applicant is required under 35 U.S.C. 121 to elect a single disclosed species for prosecution on the merits to which the claims shall be restricted if no generic claim is finally held to be allowable. Currently, claims 43-46, 53-59 are generic.

Applicant is advised that a reply to this requirement must include an identification of the species that is elected consonant with this requirement, and a listing of all claims readable thereon, including any claims subsequently added. An argument that a claim is allowable or that all claims are generic is considered nonresponsive unless accompanied by an election.

Upon the allowance of a generic claim, applicant will be entitled to consideration of claims to additional species which are written in dependent form or otherwise include all the limitations of an allowed generic claim as provided by 37 CFR 1.141. If claims are added after the election, applicant must indicate which are readable upon the elected species. MPEP § 809.02(a).

Should applicant traverse on the ground that the species are not patentably distinct, applicant should submit evidence or identify such evidence now of record showing the species to be obvious variants or clearly admit on the record that this is the case. In either instance, if the examiner finds one of the inventions unpatentable over the prior art, the evidence or admission may be used in a rejection under 35 U.S.C. 103(a) of the other invention.

3. Because these inventions are distinct for the reasons given above and have acquired a separate status in the art because of their recognized divergent subject matter and different classifications, restriction for examination purposes as indicated is proper.

4. Applicant is reminded that upon the cancellation of claims to a non-elected invention, the inventorship must be amended in compliance with 37 CFR 1.48(b) if one or more of the currently named inventors is no longer an inventor of at least one claim remaining in the application. Any amendment of inventorship must be accompanied by a petition under 37 CFR 1.48(b) and by the fee required under 37 CFR 1.17(l).

5. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Larry R. Helms, Ph.D., whose telephone number is (703) 306-5879. The examiner can normally be reached on Monday through Friday from 7:00 am to 4:30 pm, with alternate Fridays off. If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Anthony Caputa, can be reached on (703) 308-3995. Any inquiry of a general nature or relating to the status of

this application or proceeding should be directed to the Group receptionist whose telephone number is (703) 308-0196.

6. Papers related to this application may be submitted to Group 1600 by facsimile transmission. Papers should be faxed to Group 1600 via the PTO Fax Center located in Crystal Mall 1. The faxing of such papers must conform with the notice published in the Official Gazette, 1096 OG 30 (November 15, 1989). The CM1 Fax Center telephone number is (703) 308-4242.

Respectfully,
Larry R. Helms Ph.D.
703-306-5879

EXT. (1) / #12
11.14.02

Patent Docket P1093P1D1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Manuel Baca et al. Serial No.: 09/723,752 Filed: November 27, 2000 For: ANTI-VEGF ANTIBODIES	Group Art Unit: 1642 Examiner: Helms, Larry Ronald **CERTIFICATE OF MAILING** I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Assistant Commissioner of Patents, Washington, D.C. 20231 on October 28 2002 Eileen Ly

PETITION AND FEE FOR ONE MONTH EXTENSION OF TIME
(37 CFR 1.136(a))

Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

Applicant petitions the Commissioner of Patents and Trademarks to extend the time for response to the Office Action dated August 28, 2002 for one (1) month(s) from September 28, 2002 to October 28, 2002. The extended time for response does not exceed the statutory period.

Please charge Deposit Account No. 07-0630 in the amount of $110 to cover the cost of the extension. Any deficiency or overpayment should be charged or credited to this deposit account.
A duplicate of this sheet is enclosed.

Respectfully submitted,
GENENTECH, INC.

Date: October 28 2002

By: _____
Steven X. Cui
Reg. No. 44,637
Telephone No. (650) 225-8674

09157
PATENT TRADEMARK OFFICE

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Election/Amdt C/#13
11-14-02

Patent Docket P1093P1D1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Manuel Baca et al. Serial No.: 09/723,752 Filed: November 27, 2000 For: ANTI-VEGF ANTIBODIES	Group Art Unit: 1614 Examiner: **CERTIFICATE OF MAILING** I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Assistant Commissioner of Patents, Washington, D.C. 20231 on October 28, 2002 Eileen Ly

AMENDMENT AND RESPONSE TO RESTRICTION REQUIREMENT

Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

This paper is filed in response to the Office Action mailed August 28, 2002, setting forth a restriction requirement in connection with the above-identified application. A response to the requirement is due September 28, 2002. Applicants submit concurrently herewith a Request for One Month Extension of Time along with the required fee. Accordingly, this paper is timely filed.

Please amend the application as follows and consider the remarks set forth thereafter. Attached hereto is a marked-up version of the changes made to the specification and claims by the current amendment. The attached page is captioned "**Version with markings to show changes made.**" In addition, for the Examiner's convenience, attached hereto as Appendix A is a complete set of the currently pending claims as amended.

AMENDMENT

In the Sequence Listing:

Please enter the substitute Sequence Listing submitted herewith to replace the sequence listing currently on file.

#122265

In the Speicification:

Please replace the paragraph beginning at page 7, line 15 with the following rewritten paragraph:

-- Figs. 8A-E depict the double stranded nucleotide sequence (SEQ ID NO:99) for phage-display antibody vector phMB4-19-1.6 in Example 3 and the amino acid sequences encoded thereby (SEQ ID NO's 100, 130 and 131).--

In the Claims:

Please cancel claim 48.

Please amend claims 47, 49-52 as follows:

47. (Amended) The method of claim 43, said humanized anti-VEGF antibody having a heavy chain and a light chain, wherein the heavy chain comprises a variable domain comprising the following complementarity determining region (CDR) amino acid sequences: CDRH1 (GYX_1FTX_2YGMN, wherein X_1 is T or D and X_2 is N or H; SEQ ID NO: 128), CDRH2 (WINTYTGEPTYAADFKR; SEQ ID NO:2) and CDRH3 ($YPX_1YYGX_2SHWYFDV$, wherein X_1 is Y or H and X_2 is S or T; SEQ ID NO: 129).

49. (Amended) The method of claim 47, wherein the heavy chain comprises a variable domain comprising the following CDR amino acid sequences: CDRH 1 (GYTFTNYGMN; SEQ ID NO: 1), CDRH2 (WINTYTGEPTYAADFKR; SEQ ID NO:2) and CDRH3 (YPHYYGSSHWYFDV; SEQ ID NO:3).

50. (Amended) The method of claim 47, wherein the light chain of the humanized anti-VEGF antibody comprises a variable domain comprising the following CDR amino acid sequences: CDRL1 (SASQDISNYLN; SEQ ID NO:4), CDRL2 (FTSSLHS SEQ ID NO:5) and CDRL3 (QQYSTVPWT; SEQ ID NO:6).

51. (Amended) The method of claim 43, said humanized anti-VEGF antibody comprising a heavy chain variable domain sequence of SEQ ID NO:115 and a light chain variable domain sequence of SEQ ID NO:116.

C3

52. (Amended) The method of claim 43, said humanized anti-VEGF antibody comprising a heavy chain variable domain sequence of SEQ ID NO:7 and a light chain variable domain sequence of SEQ ID NO:8.

REMARKS

I. Substitute Sequence Listing and Specification Amendment:

The substitute Sequence Listing is submitted herewith to renumber some of the sequences so that the SEQ ID Numbers match between the Sequence Listing and the specification. Specifically, the sequences previously identified in the Sequence Listing as SEQ ID NO's 101 and 102 have been renumbered as SEQ ID NO's 130 and 131. Furthermore, the specification has been amended accordingly (i.e., at page 7, line 17).

II. Claim Amendments:

Claim 48 has been canceled. Claims 47 and 49-52 have been amended. Claims 47 and 49 are amended to merely clarify the characteristics of the claimed humanized anti-VEGF antibody as having a heavy chain and a light chain, wherein the heavy chain comprises a variable domain comprising the CDRs of specific amino acid sequences. Support for the amendments can be found in the specification at, for example, page 3, line 9-18 and in the originally filed claims. Claim 50 is amended to be dependent on claim 47, wherein the light chain comprises a variable domain comprising the CDRs of specific amino acid sequences. Support for the amendment of claim 50 can be found at, for example, page 3, line 29 through page 4, line 7. Claim 51 is amended to encompass a humanized anti-VEGF antibody comprising a heavy chain variable domain of SEQ ID NO:115 and a light chain variable domain of SEQ ID NO:116. Such antibody is described at, for example, page 4, line 28 through page 5, line 5 and in Figures 10A and 10B (under "Y0317"). As such, the amendments do not add new matter.

III. Restriction Requirement:

The outstanding Office Action requires Applicants to elect a single species with respect to claims 47-52. According to the Office, claims 47-52 are directed to specific SEQ ID NO's for CDRs and for entire antibodies. Claims 43-46, 53-59 are deemed as generic.

In response to the restriction requirement, and further in light of the claim amendements, Applicants hereby elect the species encompassing antibodies having CDRs of specific sequences for further prosecution. Specifically, claims 47, 49 and 50 are readable upon the elected species. This election is made without traverse.

In the event any additional fees are due in connection with the filing of these documents, the Commissioner is authorized to charge such fees to our Deposit Account No. 07-0630.

Respectfully submitted,

GENENTECH, INC.

Date: October 28, 2002 By: _____

Steven X. Cui
Reg. No. 44,637
Telephone No. (650) 225-8674

VERSION WITH MARKINGS TO SHOW CHANGES MADE

In the Specification:

Paragraph beginning at page 7, line 15 has been amended as follows:

Figs. 8A-E depict the double stranded nucleotide sequence (SEQ ID NO:99) for phage-display antibody vector phMB4-19-1.6 in Example 3 and the amino acid sequences encoded thereby (SEQ ID NO's 100, 130 and 131).

In the Claims:

Claim 48 has been canceled. Claims 47, 49-52 have been amended as follows:

47. (Amended) The method of claim 43, said humanized anti-VEGF antibody having a heavy chain and a light chain, wherein the heavy chain comprises a variable domain comprising the following [hypervariable region] complementarity determining region (CDR) amino acid sequences: CDRH1 (GYX_1FTX_2YGMN, wherein X_1 is T or D and X_2 is N or H; SEQ ID NO:128), CDRH2 (WINTYTGEPTYAADFKR; SEQ ID NO:2) and CDRH3 ($YPX_1YYGX_2SHWYFDV$, wherein X_1 is Y or H and X_2 is S or T; SEQ ID NO:129).

49. (Amended) The method of claim 47, [said humanized anti-VEGF antibody having a] wherein the heavy chain comprises a variable domain comprising the following [hypervariable region] CDR amino acid sequences: CDRH 1 (GYTFTNYGMN; SEQ ID NO: 1), CDRH2 (WINTYTGEPTYAADFKR; SEQ ID NO:2) and CDRH3 (YPHYYGSSHWYFDV; SEQ ID NO:3).

50. (Amended) The method of claim 47[3], [said] wherein the light chain of the humanized anti-VEGF antibody [having a light chain] comprises a variable domain comprising the following [hypervariable region] CDR amino acid sequences: CDRL1 (SASQDISNYLN; SEQ ID NO:4), CDRL2 (FTSSLHS SEQ ID NO:5) and CDRL3 (QQYSTVPWT; SEQ ID NO:6).

51. (Amended) The method of claim 43, said humanized anti-VEGF antibody comprising [the amino acid] a heavy chain variable domain sequence of SEQ ID NO:[8]115 and a light chain variable domain sequence of SEQ ID NO:116.

52. (Amended) The method of claim 43, said humanized anti-VEGF antibody [having] comprising a heavy chain variable domain [comprising the amino acid] sequence of SEQ ID NO:7 and a light chain variable domain [comprising the amino acid] sequence of SEQ ID NO:8.

Appendix A

Pending Claims

43. A method for inhibiting VEGF-induced angiogenesis in a subject, comprising administering to said subject an effective amount of a humanized anti-VEGF antibody which binds human VEGF with a K_d value of no more than about 1 x 10^{-8}M.

44. The method of claim 43, wherein said humanized anti-VEGF antibody which binds human VEGF with a K_d value of no more than about 1 x 10^{-9}M.

45. The method of claim 43, wherein said subject has a tumor.

46. The method of claim 45, wherein 5mg/kg of said humanized antibody inhibits at least about 50% of tumor growth in an A673 *in vivo* tumor model.

47. (Amended) The method of claim 43, said humanized anti-VEGF antibody having a heavy chain and a light chain, wherein the heavy chain comprises a variable domain comprsing the following complementarity determining region (CDR) amino acid sequences: CDRH1 (GYX_1FTX_2YGMN, wherein X_1 is T or D and X_2 is N or H; SEQ ID NO: 128), CDRH2 (WINTYTGEPTYAADFKR; SEQ ID NO:2) and CDRH3 ($YPX_1YYGX_2SHWYFDV$, wherein X_1 is Y or H and X_2 is S or T; SEQ ID NO: 129).

49. (Amended) The method of claim 47, wherein the heavy chain comprises a variable domain comprising the following CDR amino acid sequences: CDRH 1 (GYTFTNYGMN; SEQ ID NO: l), CDRH2 (WINTYTGEPTYAADFKR; SEQ ID NO:2) and CDRH3 (YPHYYGSSHWYFDV; SEQ ID NO:3).

50. (Amended) The method of claim 47, wherein the light chain of the humanized anti-VEGF antibody comprises a variable domain comprising the following CDR amino acid sequences: CDRL1 (SASQDISNYLN; SEQ ID NO:4), CDRL2 (FTSSLHS SEQ ID NO:5) and CDRL3 (QQYSTVPWT; SEQ ID NO:6).

51. (Amended) The method of claim 43, said humanized anti-VEGF antibody comprising a heavy chain variable domain sequence of SEQ ID NO:115 and a light chain variable domain

sequence of SEQ ID NO:116.

52. (Amended) The method of claim 43, said humanized anti-VEGF antibody comprising a heavy chain variable domain sequence of SEQ ID NO:7 and a light chain variable domain sequence of SEQ ID NO:8.

53. The method of claim 43, wherein said humanized anti-VEGF antibody is a full length antibody.

54. The method of claim 53, wherein said humanized anit-VEGF antibody is a human IgG.

55. The method of claim 43, wherein said humanized anti-VEGF antibody is an antibody fragment.

56. The method of claim 55, wherein said humanized anti-VEGF antibody is a Fab.

57. The method of claim 43, wherein said subject has a retinal disease.

58. The method of claim 57, wherein said retinal disease is age-related macular degeneration (AMD).

59. The method of claim 58, wherein the humanized anti-VEGF antibody is administered to the subject at a dose of at least about 0.5mg/kg.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Baca, Manuel
Wells, James A.
Presta, Leonard G.
Lowman, Henry B.
Chen, Yvonne M.

(ii) TITLE OF INVENTION: ANTI-VEGF ANTIBODIES

(iii) NUMBER OF SEQUENCES: 131

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Genentech, Inc.
(B) STREET: 1 DNA Way
(C) CITY: South San Francisco
(D) STATE: California
(E) COUNTRY: USA
(F) ZIP: 94080

C4

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: 3.5 inch, 1.44 Mb floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: WinPatin (Genentech)

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: 09/723752
(B) FILING DATE: 27-Nov-2000
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 08/908469
(B) FILING DATE: 06-AUG-1997

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 08/833504
(B) FILING DATE: 07-APR-1997

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Cui, Steven X.
(B) REGISTRATION NUMBER: 44,637
(C) REFERENCE/DOCKET NUMBER: P1093P1D1

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 650/225-8674
(B) TELEFAX: 650/952-9881

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Gly Tyr Thr Phe Thr Asn Tyr Gly Met Asn
1 5 10

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Trp Ile Asn Thr Tyr Thr Gly Glu Pro Thr Tyr Ala Ala Asp Phe
1 5 10 15

Lys Arg

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Tyr Pro His Tyr Tyr Gly Ser Ser His Trp Tyr Phe Asp Val
1 5 10

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Ser Ala Ser Gln Asp Ile Ser Asn Tyr Leu Asn
1 5 10

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Phe Thr Ser Ser Leu His Ser
1 5

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Gln Gln Tyr Ser Thr Val Pro Trp Thr
1 5

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 118 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
1 5 10 15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr
20 25 30

Asn Tyr Gly Met Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
35 40 45

Glu Trp Val Gly Trp Ile Asn Thr Tyr Thr Gly Glu Pro Thr Tyr
50 55 60

Ala Ala Asp Phe Lys Arg Arg Phe Thr Phe Ser Leu Asp Thr Ser
65 70 75

Lys Ser Thr Ala Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
80 85 90

```
Thr Ala Val Tyr Tyr Cys Ala Lys Tyr Pro His Tyr Tyr Gly Ser
                 95                  100                 105

Ser His Trp Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu
                110                 115
```

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 110 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
 1               5                  10                  15

Gly Asp Arg Val Thr Ile Thr Cys Ser Ala Ser Gln Asp Ile Ser
                20                  25                  30

Asn Tyr Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
                35                  40                  45

Val Leu Ile Tyr Phe Thr Ser Ser Leu His Ser Gly Val Pro Ser
                50                  55                  60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
                65                  70                  75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
                80                  85                  90

Tyr Ser Thr Val Pro Trp Thr Phe Gly Gln Gly Thr Lys Val Glu
                95                 100                 105

Ile Lys Arg Thr Val
               110
```

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 123 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```
Glu Ile Gln Leu Val Gln Ser Gly Pro Glu Leu Lys Gln Pro Gly
 1               5                  10                  15
```

```
Glu Thr Val Arg Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr
                  20                  25                  30

Asn Tyr Gly Met Asn Trp Val Lys Gln Ala Pro Gly Lys Gly Leu
                  35                  40                  45

Lys Trp Met Gly Trp Ile Asn Thr Tyr Thr Gly Glu Pro Thr Tyr
                  50                  55                  60

Ala Ala Asp Phe Lys Arg Arg Phe Thr Phe Ser Leu Glu Thr Ser
                  65                  70                  75

Ala Ser Thr Ala Tyr Leu Gln Ile Ser Asn Leu Lys Asn Asp Asp
                  80                  85                  90

Thr Ala Thr Tyr Phe Cys Ala Lys Tyr Pro His Tyr Tyr Gly Ser
                  95                 100                 105

Ser His Trp Tyr Phe Asp Val Trp Gly Ala Gly Thr Thr Val Thr
                 110                 115                 120

Val Ser Ser
```

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 108 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
Asp Ile Gln Met Thr Gln Thr Thr Ser Ser Leu Ser Ala Ser Leu
  1               5                  10                  15

Gly Asp Arg Val Ile Ile Ser Cys Ser Ala Ser Gln Asp Ile Ser
                  20                  25                  30

Asn Tyr Leu Asn Trp Tyr Gln Gln Lys Pro Asp Gly Thr Val Lys
                  35                  40                  45

Val Leu Ile Tyr Phe Thr Ser Ser Leu His Ser Gly Val Pro Ser
                  50                  55                  60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Tyr Ser Leu Thr Ile
                  65                  70                  75

Ser Asn Leu Glu Pro Glu Asp Ile Ala Thr Tyr Tyr Cys Gln Gln
                  80                  85                  90
```

```
Tyr Ser Thr Val Pro Trp Thr Phe Gly Gly Gly Thr Lys Leu Glu
                 95                  100                 105

Ile Lys Arg
```

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 113 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

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Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
  1               5                  10                  15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser
                 20                  25                  30

Ser Tyr Ala Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
                 35                  40                  45

Glu Trp Val Ser Val Ile Ser Gly Asp Gly Gly Ser Thr Tyr Tyr
                 50                  55                  60

Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser
                 65                  70                  75

Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
                 80                  85                  90

Thr Ala Val Tyr Tyr Cys Ala Arg Gly Phe Asp Tyr Trp Gly Gln
                 95                 100                 105

Gly Thr Leu Val Thr Val Ser Ser
                110
```

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 108 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
  1               5                  10                  15
```

```
Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Ser Ile Ser
                  20                  25                  30

Asn Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
                  35                  40                  45

Leu Leu Ile Tyr Ala Ala Ser Ser Leu Glu Ser Gly Val Pro Ser
                  50                  55                  60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
                  65                  70                  75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
                  80                  85                  90

Tyr Asn Ser Leu Pro Trp Thr Phe Gly Gln Gly Thr Lys Val Glu
                  95                 100                 105

Ile Lys Arg
```

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 107 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

```
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
  1               5                  10                  15

Gly Asp Arg Val Thr Ile Thr Cys Ser Ala Ser Gln Asp Ile Ser
                  20                  25                  30

Asn Tyr Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
                  35                  40                  45

Leu Leu Ile Tyr Phe Thr Ser Ser Leu His Ser Gly Val Pro Ser
                  50                  55                  60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
                  65                  70                  75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
                  80                  85                  90

Tyr Ser Thr Val Pro Trp Thr Phe Gly Gln Gly Thr Lys Val Glu
                  95                 100                 105
```

Ile Lys

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 123 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

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Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
 1               5                  10                  15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr
            20                  25                  30

Asn Tyr Gly Met Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
            35                  40                  45

Glu Trp Val Gly Trp Ile Asn Thr Tyr Thr Gly Glu Pro Thr Tyr
            50                  55                  60

Ala Ala Asp Phe Lys Arg Arg Phe Thr Ile Ser Arg Asp Asn Ser
            65                  70                  75

Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
            80                  85                  90

Thr Ala Val Tyr Tyr Cys Ala Arg Tyr Pro His Tyr Tyr Gly Ser
            95                 100                 105

Ser His Trp Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu Val Thr
           110                 115                 120

Val Ser Ser
```

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 107 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

```
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
 1               5                  10                  15
```

```
Gly Asp Arg Val Thr Ile Thr Cys Ser Ala Ser Gln Asp Ile Ser
                  20                  25                  30

Asn Tyr Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
                  35                  40                  45

Leu Leu Ile Tyr Phe Thr Ser Ser Leu His Ser Gly Val Pro Ser
                  50                  55                  60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Tyr Thr Leu Thr Ile
                  65                  70                  75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
                  80                  85                  90

Tyr Ser Thr Val Pro Trp Thr Phe Gly Gln Gly Thr Lys Val Glu
                  95                 100                 105

Ile Lys
```

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 123 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

```
Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
  1               5                  10                  15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr
                  20                  25                  30

Asn Tyr Gly Met Asn Trp Ile Arg Gln Ala Pro Gly Lys Gly Leu
                  35                  40                  45

Glu Trp Val Gly Trp Ile Asn Thr Tyr Thr Gly Glu Pro Thr Tyr
                  50                  55                  60

Ala Ala Asp Phe Lys Arg Arg Phe Thr Ile Ser Leu Asp Thr Ser
                  65                  70                  75

Ala Ser Thr Val Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
                  80                  85                  90

Thr Ala Val Tyr Tyr Cys Ala Lys Tyr Pro His Tyr Tyr Gly Ser
                  95                 100                 105
```

Ser His Trp Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu Val Thr
110 115 120

Val Ser Ser

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Pro Lys Asn Ser Ser Met Ile Ser Asn Thr Pro
1 5 10

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

His Gln Ser Leu Gly Thr Gln
1 5

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

His Gln Asn Leu Ser Asp Gly Lys
1 5

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

His Gln Asn Ile Ser Asp Gly Lys
1 5

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Val Ile Ser Ser His Leu Gly Gln
1 5

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 66 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

GATTTCAAAC GTCGTNYTAC TWTTTCTAGA GACAACTCCA AAAACACABY 50

TTACCTGCAG ATGAAC 66

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 66 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

GATTTCAAAC GTCGTNYTAC TWTTTCTTTA GACACCTCCG CAAGCACABY 50

TTACCTGCAG ATGAAC 66

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

AGCCTGCGCG CTGAGGACAC TGCCGTCTAT TACTGTDYAA RGTACCCCCA 50

CTATTATGGG 60

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

CTCAGCGCGC AGGCTGTTCA TCTGCAGGTA 30

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

GCTGATATCC AGTTGACCCA GTCCCCG 27

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

TCTGGGACGG ATTACACTCT GACCATC 27

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 75 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

CGTTTGTCCT GTGCARYTTC TGGCTATACC TTCACCAACT ATGGTATGAA 50

CTGGRTCCGT CAGGCCCCGG GTAAG 75

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

GATATCCAGT TGACCCAGTC CCCG 24

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

GCTCCGAAAG TACTGATTTA C 21

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 54 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

CGTCGTTTCA CTTTTTCTGC AGACACCTCC AGCAACACAG TATACCTGCA 50

GATG 54

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

CTATTACTGT GCAAAGTACC CCCAC 25

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

GGGACGGATT TCACTCTGAC CATC 24

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

GGTATGAACT GGGTCCGTCA GGCCCC 26

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 57 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

CGTCGTTTCA CTTTTTCTTT AGACACCTCC AAAAGCACAG CATACCTGCA 50

GATGAAC 57

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 53 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

GGGTCACCAT CACCTGCTAA GCATAATAAT AATAAAGCAA CTATTTAAAC 50

TGG 53

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 52 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

GCGCAAGTCA GGATATTTAA TAATAATAAT AATGGTATCA ACAGAAACCA 50

GG 52

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 48 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

GTCTATTACT GTGCAAAGTA ATAACACTAA TAAGGGAGCA GCCACTGG 48

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 49 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

GGTACCCCCA CTATTATTAA TAATAATAAT GGTATTTCGA CGTCTGGGG 49

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 53 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

CACTATTATG GGAGCAGCCA CTAATAATAA TAAGTCTGGG TCAAGGAACC 50

CTG 53

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 53 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

TCCTGTGCAG CTTCTGGCTA ATAATTCTAA TAATAAGGTA TGAACTGGGT 50

CCG 53

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 52 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

GAATGGGTTG GATGGATTAA CTAATAATAA GGTTAACCGA CCTATGCTGC 50

GG 52

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 49 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

CTGTGCAAAG TACCCGTAAT ATTAATAATA ATAACACTGG TATTTCGAC 49

(2) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 48 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

CGTTTCACTT TTTCTTAAGA CTAATCCAAA TAAACAGCAT ACCTGCAG 48

(2) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 46 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

GAATGGGTTG GATGGATTTA ATAATAATAA GGTGAACCGA CCTATG 46

(2) INFORMATION FOR SEQ ID NO:46:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 53 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

GGGTCACCAT CACCTGCNNS GCANNSNNSN NSNNSAGCAA CTATTTAAAC 50

TGG 53

(2) INFORMATION FOR SEQ ID NO:47:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 52 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

GCGCAAGTCA GGATATTNNS NNSNNSNNSN NSTGGTATCA ACAGAAACCA 50

GG 52

(2) INFORMATION FOR SEQ ID NO:48:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 48 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

GTCTATTACT GTGCAAAGNN SNNSCACNNS NNSGGGAGCA GCCACTGG 48

(2) INFORMATION FOR SEQ ID NO:49:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 49 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

GGTACCCCCA CTATTATNNS NNSNNSNNST GGTATTTCGA CGTCTGGGG 49

(2) INFORMATION FOR SEQ ID NO:50:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 54 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

CACTATTATG GGAGCAGCCA CNNSNNSNNS NNSGTCTGGG GTCAAGGAAC 50

CCTG 54

(2) INFORMATION FOR SEQ ID NO:51:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 53 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

TCCTGTGCAG CTTCTGGCNN SNNSTTCNNS NNSNNSGGTA TGAACTGGGT 50

CCG 53

(2) INFORMATION FOR SEQ ID NO:52:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 52 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:

GAATGGGTTG GATGGATTAA CNNSNNSNNS GGTNNSCCGA CCTATGCTGC 50

GG 52

(2) INFORMATION FOR SEQ ID NO:53:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 49 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:53:

CTGTGCAAAG TACCCGNNST ATNNSNNSNN SNNSCACTGG TATTTCGAC 49

(2) INFORMATION FOR SEQ ID NO:54:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 48 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:54:

CGTTTCACTT TTTCTNNSGA CNNSTCCAAA NNSACAGCAT ACCTGCAG 48

(2) INFORMATION FOR SEQ ID NO:55:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 46 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:55:

GAATGGGTTG GATGGATTNN SNNSNNSNNS GGTGAACCGA CCTATG 46

(2) INFORMATION FOR SEQ ID NO:56:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:56:

Tyr Pro Tyr Tyr Arg Gly Thr Ser His Trp Tyr Phe Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:57:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:57:

Tyr Pro Tyr Tyr Ile Asn Lys Ser His Trp Tyr Phe Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:58:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:58:

Tyr Pro Tyr Tyr Tyr Gly Thr Ser His Trp Tyr Phe Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:59:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:59:

Tyr Pro Tyr Tyr Tyr Asn Gln Ser His Trp Tyr Phe Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:60:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:60:

Tyr Pro Tyr Tyr Ile Ala Lys Ser His Trp Tyr Phe Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:61:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:61:

Tyr Pro Tyr Tyr Arg Asp Asn Ser His Trp Tyr Phe Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:62:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:62:

Tyr Pro Tyr Tyr Trp Gly Thr Ser His Trp Tyr Phe Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:63:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:63:

Tyr Pro Tyr Tyr Arg Gln Asn Ser His Trp Tyr Phe Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:64:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:64:

Tyr Pro Tyr Tyr Arg Gln Ser Ser His Trp Tyr Phe Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:65:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:65:

Tyr Pro Tyr Tyr Arg Asn Thr Ser His Trp Tyr Phe Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:66:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 13 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:66:

Tyr Pro Tyr Tyr Lys Asn Thr Ser His Trp Tyr Phe Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:67:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:67:

Tyr Pro Tyr Tyr Ile Glu Arg Ser His Trp Tyr Phe Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:68:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:68:

Tyr Pro Tyr Tyr Arg Asn Ala Ser His Trp Tyr Phe Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:69:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:69:

Tyr Pro Tyr Tyr Thr Thr Arg Ser His Trp Tyr Phe Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:70:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:70:

Tyr Pro Tyr Tyr Glu Gly Ser Ser His Trp Tyr Phe Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:71:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:71:

Tyr Pro Tyr Tyr Arg Gln Arg Gly His Trp Tyr Phe Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:72:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:72:

Tyr Pro Tyr Tyr Thr Gly Arg Ser His Trp Tyr Phe Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:73:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:73:

Tyr Pro Tyr Tyr Thr Asn Thr Ser His Trp Tyr Phe Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:74:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:74:

Tyr Pro Tyr Tyr Arg Lys Gly Ser His Trp Tyr Phe Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:75:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:75:

Tyr Pro Tyr Tyr Thr Gly Ser Ser His Trp Tyr Phe Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:76:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:76:

Tyr Pro Tyr Tyr Arg Ser Gly Ser His Trp Tyr Phe Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:77:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:77:

Tyr Pro Tyr Tyr Thr Asn Arg Ser His Trp Tyr Phe Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:78:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:78:

Tyr Pro Tyr Tyr Arg Asn Ser Ser His Trp Tyr Phe Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:79:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:79:

Tyr Pro Tyr Tyr Lys Glu Ser Ser His Trp Tyr Phe Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:80:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:80:

Tyr Pro Tyr Tyr Arg Asp Ala Ser His Trp Tyr Phe Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:81:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:81:

Tyr Pro Tyr Tyr Arg Gln Lys Gly His Trp Tyr Phe Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:82:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:82:

Tyr Pro Tyr Tyr Lys Gly Gly Ser His Trp Tyr Phe Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:83:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 13 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:83:

Tyr Pro Tyr Tyr Tyr Gly Ala Ser His Trp Tyr Phe Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:84:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:84:

Tyr Pro Tyr Tyr Arg Gly Glu Ser His Trp Tyr Phe Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:85:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:85:

Tyr Pro Tyr Tyr Arg Ser Thr Ser His Trp Tyr Phe Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:86:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:86:

Gly Tyr Asp Phe Thr His Tyr Gly Met Asn
1 5 10

(2) INFORMATION FOR SEQ ID NO:87:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:87:

Gly Tyr Glu Phe Gln His Tyr Gly Met Asn
1 5 10

(2) INFORMATION FOR SEQ ID NO:88:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:88:

Gly Tyr Glu Phe Thr His Tyr Gly Met Asn
1 5 10

(2) INFORMATION FOR SEQ ID NO:89:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:89:

Gly Tyr Asp Phe Gly His Tyr Gly Met Asn
1 5 10

(2) INFORMATION FOR SEQ ID NO:90:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:90:

Gly Tyr Asp Phe Ser His Tyr Gly Met Asn
1 5 10

(2) INFORMATION FOR SEQ ID NO:91:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:91:

Gly Tyr Glu Phe Ser His Tyr Gly Met Asn
1 5 10

(2) INFORMATION FOR SEQ ID NO:92:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:92:

Phe Ser Val Asp Val Ser Lys Ser Thr Ala
1 5 10

(2) INFORMATION FOR SEQ ID NO:93:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:93:

Phe Ser Leu Asp Lys Ser Lys Ser Thr Ala
1 5 10

(2) INFORMATION FOR SEQ ID NO:94:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:94:

Phe Ser Leu Asp Val Trp Lys Ser Thr Ala
1 5 10

(2) INFORMATION FOR SEQ ID NO:95:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:95:

Phe Ser Ile Asp Lys Ser Lys Ser Thr Ala
1 5 10

(2) INFORMATION FOR SEQ ID NO:96:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:96:

GCAAAGTACC CGTACTATTA TGGGACGAGC CACTGGTATT TC 42

(2) INFORMATION FOR SEQ ID NO:97:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 48 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:97:

GTCACCATCA CCTGCAGCGC AAGTCAGGAT ATTAGCAACT ATTTAAAC 48

(2) INFORMATION FOR SEQ ID NO:98:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:98:

CCGTACTATT ATGGGAGCAG CCACTGGTAT TTC 33

(2) INFORMATION FOR SEQ ID NO:99:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6072 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:99:

GAATTCAACT TCTCCATACT TTGGATAAGG AAATACAGAC ATGAAAAATC 50

TCATTGCTGA GTTGTTATTT AAGCTTTGGA GATTATCGTC ACTGCAATGC 100

TTCGCAATAT GGCGCAAAAT GACCAACAGC GGTTGATTGA TCAGGTAGAG 150

GGGGCGCTGT ACGAGGTAAA GCCCGATGCC AGCATTCCTG ACGACGATAC 200

GGAGCTGCTG CGCGATTACG TAAAGAAGTT ATTGAAGCAT CCTCGTCAGT 250

AAAAAGTTAA TCTTTTCAAC AGCTGTCATA AAGTTGTCAC GGCCGAGACT 300

TATAGTCGCT TTGTTTTTAT TTTTTAATGT ATTTGTAACT AGAATTCGAG 350

CTCGGTACCC GGGGATCCTC TAGAGGTTGA GGTGATTTTA TGAAAAAGAA 400

TATCGCATTT CTTCTTGCAT CTATGTTCGT TTTTTCTATT GCTACAAACG 450

CGTACGCTGA TATCCAGTTG ACCCAGTCCC CGAGCTCCCT GTCCGCCTCT 500

GTGGGCGATA GGGTCACCAT CACCTGCAGC GCAAGTCAGG ATATTAGCAA 550

CTATTTAAAC TGGTATCAAC AGAAACCAGG AAAAGCTCCG AAACTACTGA 600

TTTACTTCAC CTCCTCTCTC CACTCTGGAG TCCCTTCTCG CTTCTCTGGA 650

TCCGGTTCTG GGACGGATTA CACTCTGACC ATCAGCAGTC TGCAGCCAGA 700

AGACTTCGCA ACTTATTACT GTCAACAGTA TAGCACCGTG CCGTGGACGT 750

TTGGACAGGG TACCAAGGTG GAGATCAAAC GAACTGTGGC TGCACCATCT 800

GTCTTCATCT TCCCGCCATC TGATGAGCAG TTGAAATCTG GAACTGCTTC 850

TGTTGTGTGC CTGCTGAATA ACTTCTATCC CAGAGAGGCC AAAGTACAGT 900

GGAAGGTGGA TAACGCCCTC CAATCGGGTA ACTCCCAGGA GAGTGTCACA 950

GAGCAGGACA GCAAGGACAG CACCTACAGC CTCAGCAGCA CCCTGACGCT 1000

GAGCAAAGCA GACTACGAGA AACACAAAGT CTACGCCTGC GAAGTCACCC 1050

ATCAGGGCCT GAGCTCGCCC GTCACAAAGA GCTTCAACAG GGGAGAGTGT 1100

TAAGCTGATC CTCTACGCCG GACGCATCGT GGCCCTAGTA CGCAACTAGT 1150

CGTAAAAAGG GTATCTAGAG GTTGAGGTGA TTTTATGAAA AAGAATATCG 1200

CATTTCTTCT TGCATCTATG TTCGTTTTTT CTATTGCTAC AAACGCGTAC 1250

GCTGAGGTTC AGCTGGTGGA GTCTGGCGGT GGCCTGGTGC AGCCAGGGGG 1300

CTCACTCCGT TTGTCCTGTG CAGCTTCTGG CTATACCTTC ACCAACTATG 1350

GTATGAACTG GATCCGTCAG GCCCCGGGTA AGGGCCTGGA ATGGGTTGGA 1400

TGGATTAACA CCTATACCGG TGAACCGACC TATGCTGCGG ATTTCAAACG 1450

TCGTTTTACT ATATCTGCAG ACACCTCCAG CAACACAGTT TACCTGCAGA 1500

TGAACAGCCT GCGCGCTGAG GACACTGCCG TCTATTACTG TGCAAAGTAC 1550

CCGCACTATT ATGGGAGCAG CCACTGGTAT TTCGACGTCT GGGGTCAAGG 1600

AACCCTGGTC ACCGTCTCCT CGGCCTCCAC CAAGGGCCCA TCGGTCTTCC 1650

CCCTGGCACC CTCCTCCAAG AGCACCTCTG GGGGCACAGC GGCCCTGGGC 1700

TGCCTGGTCA AGGACTACTT CCCCGAACCG GTGACGGTGT CGTGGAACTC 1750

AGGCGCCCTG ACCAGCGGCG TGCACACCTT CCCGGCTGTC CTACAGTCCT 1800

CAGGACTCTA CTCCCTCAGC AGCGTGGTGA CCGTGCCCTC CAGCAGCTTG 1850

GGCACCCAGA CCTACATCTG CAACGTGAAT CACAAGCCCA GCAACACCAA 1900

GGTCGACAAG AAAGTTGAGC CCAAATCTTG TGACAAAACT CACCTCTAGA 1950

GTGGCGGTGG CTCTGGTTCC GGTGATTTTG ATTATGAAAA GATGGCAAAC 2000

GCTAATAAGG GGGCTATGAC CGAAAATGCC GATGAAAACG CGCTACAGTC 2050

TGACGCTAAA GGCAAACTTG ATTCTGTCGC TACTGATTAC GGTGCTGCTA 2100

TCGATGGTTT CATTGGTGAC GTTTCCGGCC TTGCTAATGG TAATGGTGCT 2150

ACTGGTGATT TTGCTGGCTC TAATTCCCAA ATGGCTCAAG TCGGTGACGG 2200

TGATAATTCA CCTTTAATGA ATAATTTCCG TCAATATTTA CCTTCCCTCC 2250

CTCAATCGGT TGAATGTCGC CCTTTTGTCT TTAGCGCTGG TAAACCATAT 2300

GAATTTTCTA TTGATTGTGA CAAAATAAAC TTATTCCGTG GTGTCTTTGC 2350

GTTTCTTTTA TATGTTGCCA CCTTTATGTA TGTATTTTCT ACGTTTGCTA 2400

ACATACTGCG TAATAAGGAG TCTTAATCAT GCCAGTTCTT TTGGCTAGCG 2450

CCGCCCTATA CCTTGTCTGC CTCCCCGCGT TGCGTCGCGG TGCATGGAGC 2500

CGGGCCACCT CGACCTGAAT GGAAGCCGGC GGCACCTCGC TAACGGATTC 2550

ACCACTCCAA GAATTGGAGC CAATCAATTC TTGCGGAGAA CTGTGAATGC 2600

GCAAACCAAC CCTTGGCAGA ACATATCCAT CGCGTCCGCC ATCTCCAGCA 2650

GCCGCACGCG GCGCATCTCG GGCAGCGTTG GGTCCTGGCC ACGGGTGCGC 2700

ATGATCGTGC TCCTGTCGTT GAGGACCCGG CTAGGCTGGC GGGGTTGCCT 2750

TACTGGTTAG CAGAATGAAT CACCGATACG CGAGCGAACG TGAAGCGACT 2800

GCTGCTGCAA AACGTCTGCG ACCTGAGCAA CAACATGAAT GGTCTTCGGT 2850

TTCCGTGTTT CGTAAAGTCT GGAAACGCGG AAGTCAGCGC CCTGCACCAT 2900

TATGTTCCGG ATCTGCATCG CAGGATGCTG CTGGCTACCC TGTGGAACAC 2950

CTACATCTGT ATTAACGAAG CGCTGGCATT GACCCTGAGT GATTTTTCTC 3000

TGGTCCCGCC GCATCCATAC CGCCAGTTGT TTACCCTCAC AACGTTCCAG 3050

TAACCGGGCA TGTTCATCAT CAGTAACCCG TATCGTGAGC ATCCTCTCTC 3100

GTTTCATCGG TATCATTACC CCCATGAACA GAAATTCCCC CTTACACGGA 3150

GGCATCAAGT GACCAAACAG GAAAAAACCG CCCTTAACAT GGCCCGCTTT 3200

ATCAGAAGCC AGACATTAAC GCTTCTGGAG AAACTCAACG AGCTGGACGC 3250

GGATGAACAG GCAGACATCT GTGAATCGCT TCACGACCAC GCTGATGAGC 3300

TTTACCGCAG GATCCGGAAA TTGTAAACGT TAATATTTTG TTAAAATTCG 3350

CGTTAAATTT TTGTTAAATC AGCTCATTTT TTAACCAATA GGCCGAAATC 3400

GGCAAAATCC CTTATAAATC AAAAGAATAG ACCGAGATAG GGTTGAGTGT 3450

TGTTCCAGTT TGGAACAAGA GTCCACTATT AAAGAACGTG GACTCCAACG 3500

TCAAAGGGCG AAAAACCGTC TATCAGGGCT ATGGCCCACT ACGTGAACCA 3550

TCACCCTAAT CAAGTTTTTT GGGGTCGAGG TGCCGTAAAG CACTAAATCG 3600

GAACCCTAAA GGGAGCCCCC GATTTAGAGC TTGACGGGGA AAGCCGGCGA 3650

ACGTGGCGAG AAAGGAAGGG AAGAAAGCGA AAGGAGCGGG CGCTAGGGCG 3700

CTGGCAAGTG TAGCGGTCAC GCTGCGCGTA ACCACCACAC CCGCCGCGCT 3750

TAATGCGCCG CTACAGGGCG CGTCCGGATC CTGCCTCGCG CGTTTCGGTG 3800

ATGACGGTGA AAACCTCTGA CACATGCAGC TCCCGGAGAC GGTCACAGCT 3850

TGTCTGTAAG CGGATGCCGG GAGCAGACAA GCCCGTCAGG GCGCGTCAGC 3900

GGGTGTTGGC GGGTGTCGGG GCGCAGCCAT GACCCAGTCA CGTAGCGATA 3950

GCGGAGTGTA TACTGGCTTA ACTATGCGGC ATCAGAGCAG ATTGTACTGA 4000

GAGTGCACCA TATGCGGTGT GAAATACCGC ACAGATGCGT AAGGAGAAAA 4050

TACCGCATCA GGCGCTCTTC CGCTTCCTCG CTCACTGACT CGCTGCGCTC 4100

GGTCGTTCGG CTGCGGCGAG CGGTATCAGC TCACTCAAAG GCGGTAATAC 4150

GGTTATCCAC AGAATCAGGG GATAACGCAG GAAAGAACAT GTGAGCAAAA 4200

GGCCAGCAAA AGGCCAGGAA CCGTAAAAAG GCCGCGTTGC TGGCGTTTTT 4250

CCATAGGCTC CGCCCCCCTG ACGAGCATCA CAAAAATCGA CGCTCAAGTC 4300

AGAGGTGGCG AAACCCGACA GGACTATAAA GATACCAGGC GTTTCCCCCT 4350

GGAAGCTCCC TCGTGCGCTC TCCTGTTCCG ACCCTGCCGC TTACCGGATA 4400

CCTGTCCGCC TTTCTCCCTT CGGGAAGCGT GGCGCTTTCT CATAGCTCAC 4450

GCTGTAGGTA TCTCAGTTCG GTGTAGGTCG TTCGCTCCAA GCTGGGCTGT 4500

GTGCACGAAC CCCCCGTTCA GCCCGACCGC TGCGCCTTAT CCGGTAACTA 4550

TCGTCTTGAG TCCAACCCGG TAAGACACGA CTTATCGCCA CTGGCAGCAG 4600

CCACTGGTAA CAGGATTAGC AGAGCGAGGT ATGTAGGCGG TGCTACAGAG 4650

TTCTTGAAGT GGTGGCCTAA CTACGGCTAC ACTAGAAGGA CAGTATTTGG 4700

TATCTGCGCT CTGCTGAAGC CAGTTACCTT CGGAAAAAGA GTTGGTAGCT 4750

CTTGATCCGG CAAACAAACC ACCGCTGGTA GCGGTGGTTT TTTTGTTTGC 4800

AAGCAGCAGA TTACGCGCAG AAAAAAAGGA TCTCAAGAAG ATCCTTTGAT 4850

CTTTTCTACG GGGTCTGACG CTCAGTGGAA CGAAAACTCA CGTTAAGGGA 4900

TTTTGGTCAT GAGATTATCA AAAAGGATCT TCACCTAGAT CCTTTTAAAT 4950

TAAAAATGAA GTTTTAAATC AATCTAAAGT ATATATGAGT AAACTTGGTC 5000

TGACAGTTAC CAATGCTTAA TCAGTGAGGC ACCTATCTCA GCGATCTGTC 5050

TATTTCGTTC ATCCATAGTT GCCTGACTCC CCGTCGTGTA GATAACTACG 5100

ATACGGGAGG GCTTACCATC TGGCCCCAGT GCTGCAATGA TACCGCGAGA 5150

CCCACGCTCA CCGGCTCCAG ATTTATCAGC AATAAACCAG CCAGCCGGAA 5200

GGGCCGAGCG CAGAAGTGGT CCTGCAACTT TATCCGCCTC CATCCAGTCT 5250

ATTAATTGTT GCCGGGAAGC TAGAGTAAGT AGTTCGCCAG TTAATAGTTT 5300

GCGCAACGTT GTTGCCATTG CTGCAGGCAT CGTGGTGTCA CGCTCGTCGT 5350

TTGGTATGGC TTCATTCAGC TCCGGTTCCC AACGATCAAG GCGAGTTACA 5400

TGATCCCCCA TGTTGTGCAA AAAAGCGGTT AGCTCCTTCG GTCCTCCGAT 5450

CGTTGTCAGA AGTAAGTTGG CCGCAGTGTT ATCACTCATG GTTATGGCAG 5500

CACTGCATAA TTCTCTTACT GTCATGCCAT CCGTAAGATG CTTTTCTGTG 5550

ACTGGTGAGT ACTCAACCAA GTCATTCTGA GAATAGTGTA TGCGGCGACC 5600

GAGTTGCTCT TGCCCGGCGT CAACACGGGA TAATACCGCG CCACATAGCA 5650

GAACTTTAAA AGTGCTCATC ATTGGAAAAC GTTCTTCGGG GCGAAAACTC 5700

TCAAGGATCT TACCGCTGTT GAGATCCAGT TCGATGTAAC CCACTCGTGC 5750

ACCCAACTGA TCTTCAGCAT CTTTTACTTT CACCAGCGTT TCTGGGTGAG 5800

CAAAAACAGG AAGGCAAAAT GCCGCAAAAA AGGGAATAAG GGCGACACGG 5850

AAATGTTGAA TACTCATACT CTTCCTTTTT CAATATTATT GAAGCATTTA 5900

TCAGGGTTAT TGTCTCATGA GCGGATACAT ATTTGAATGT ATTTAGAAAA 5950

ATAAACAAAT AGGGGTTCCG CGCACATTTC CCCGAAAAGT GCCACCTGAC 6000

GTCTAAGAAA CCATTATTAT CATGACATTA ACCTATAAAA ATAGGCGTAT 6050

CACGAGGCCC TTTCGTCTTC AA 6072

(2) INFORMATION FOR SEQ ID NO:100:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 237 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:100:

Met Lys Lys Asn Ile Ala Phe Leu Leu Ala Ser Met Phe Val Phe
-23 -20 -15 -10

```
Ser Ile Ala Thr Asn Ala Tyr Ala Asp Ile Gln Leu Thr Gln Ser
            -5                   1               5

Pro Ser Ser Leu Ser Ala Ser Val Gly Asp Arg Val Thr Ile Thr
         10                  15                  20

Cys Ser Ala Ser Gln Asp Ile Ser Asn Tyr Leu Asn Trp Tyr Gln
         25                  30                  35

Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile Tyr Phe Thr Ser
         40                  45                  50

Ser Leu His Ser Gly Val Pro Ser Arg Phe Ser Gly Ser Gly Ser
         55                  60                  65

Gly Thr Asp Tyr Thr Leu Thr Ile Ser Ser Leu Gln Pro Glu Asp
         70                  75                  80

Phe Ala Thr Tyr Tyr Cys Gln Gln Tyr Ser Thr Val Pro Trp Thr
         85                  90                  95

Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr Val Ala Ala
        100                 105                 110

Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser
        115                 120                 125

Gly Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg
        130                 135                 140

Glu Ala Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly
        145                 150                 155

Asn Ser Gln Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr
        160                 165                 170

Tyr Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu
        175                 180                 185

Lys His Lys Val Tyr Ala Cys Glu Val Thr His Gln Gly Leu Ser
        190                 195                 200

Ser Pro Val Thr Lys Ser Phe Asn Arg Gly Glu Cys
        205                 210
```

(2) INFORMATION FOR SEQ ID NO:101:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 110 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:101:

```
Asp Ile Gln Leu Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
 1               5                  10                  15

Gly Asp Arg Val Thr Ile Thr Cys Ser Ala Ser Gln Asp Ile Ser
            20                  25                  30

Asn Tyr Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
            35                  40                  45

Leu Leu Ile Tyr Phe Thr Ser Ser Leu His Ser Gly Val Pro Ser
            50                  55                  60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Tyr Thr Leu Thr Ile
            65                  70                  75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
            80                  85                  90

Tyr Ser Thr Val Pro Trp Thr Phe Gly Gln Gly Thr Lys Val Glu
            95                 100                 105

Ile Lys Arg Thr Val
           110
```

(2) INFORMATION FOR SEQ ID NO:102:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 118 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:102:

```
Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
 1               5                  10                  15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr
            20                  25                  30

Asn Tyr Gly Met Asn Trp Ile Arg Gln Ala Pro Gly Lys Gly Leu
            35                  40                  45

Glu Trp Val Gly Trp Ile Asn Thr Tyr Thr Gly Glu Pro Thr Tyr
            50                  55                  60

Ala Ala Asp Phe Lys Arg Arg Phe Thr Ile Ser Ala Asp Thr Ser
            65                  70                  75
```

Ser Asn Thr Val Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
80 85 90

Thr Ala Val Tyr Tyr Cys Ala Lys Tyr Pro His Tyr Tyr Gly Ser
95 100 105

Ser His Trp Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu
110 115

(2) INFORMATION FOR SEQ ID NO:103:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 110 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:103:

Asp Ile Gln Leu Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
1 5 10 15

Gly Asp Arg Val Thr Ile Thr Cys Ser Ala Ser Gln Asp Ile Ser
20 25 30

Asn Tyr Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
35 40 45

Val Leu Ile Tyr Phe Thr Ser Ser Leu His Ser Gly Val Pro Ser
50 55 60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Tyr Thr Leu Thr Ile
65 70 75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
80 85 90

Tyr Ser Thr Val Pro Trp Thr Phe Gly Gln Gly Thr Lys Val Glu
95 100 105

Ile Lys Arg Thr Val
110

(2) INFORMATION FOR SEQ ID NO:104:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 118 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:104:

```
Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
 1               5                  10                  15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr
            20                  25                  30

Asn Tyr Gly Met Asn Trp Ile Arg Gln Ala Pro Gly Lys Gly Leu
            35                  40                  45

Glu Trp Val Gly Trp Ile Asn Thr Tyr Thr Gly Glu Pro Thr Tyr
            50                  55                  60

Ala Ala Asp Phe Lys Arg Arg Phe Thr Phe Ser Ala Asp Thr Ser
            65                  70                  75

Ser Asn Thr Val Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
            80                  85                  90

Thr Ala Val Tyr Tyr Cys Ala Lys Tyr Pro His Tyr Tyr Gly Ser
            95                 100                 105

Ser His Trp Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu
           110                 115
```

(2) INFORMATION FOR SEQ ID NO:105:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 110 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:105:

```
Asp Ile Gln Leu Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
 1               5                  10                  15

Gly Asp Arg Val Thr Ile Thr Cys Ser Ala Ser Gln Asp Ile Ser
            20                  25                  30

Asn Tyr Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
            35                  40                  45

Val Leu Ile Tyr Phe Thr Ser Ser Leu His Ser Gly Val Pro Ser
            50                  55                  60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
            65                  70                  75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
            80                  85                  90
```

Tyr Ser Thr Val Pro Trp Thr Phe Gly Gln Gly Thr Lys Val Glu
95 100 105

Ile Lys Arg Thr Val
110

(2) INFORMATION FOR SEQ ID NO:106:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 118 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:106:

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
1 5 10 15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr
20 25 30

Asn Tyr Gly Met Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
35 40 45

Glu Trp Val Gly Trp Ile Asn Thr Tyr Thr Gly Glu Pro Thr Tyr
50 55 60

Ala Ala Asp Phe Lys Arg Arg Phe Thr Phe Ser Leu Asp Thr Ser
65 70 75

Lys Ser Thr Ala Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
80 85 90

Thr Ala Val Tyr Tyr Cys Ala Lys Tyr Pro His Tyr Tyr Gly Ser
95 100 105

Ser His Trp Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu
110 115

(2) INFORMATION FOR SEQ ID NO:107:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 110 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:107:

Asp Ile Gln Leu Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
1 5 10 15

```
Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Asn Glu Gln Leu Ser
                  20                  25                  30

Asn Tyr Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
                  35                  40                  45

Val Leu Ile Tyr Phe Thr Ser Ser Leu His Ser Gly Val Pro Ser
                  50                  55                  60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
                  65                  70                  75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
                  80                  85                  90

Tyr Ser Thr Val Pro Trp Thr Phe Gly Gln Gly Thr Lys Val Glu
                  95                 100                 105

Ile Lys Arg Thr Val
                 110
```

(2) INFORMATION FOR SEQ ID NO:108:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 118 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:108:

```
Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
  1               5                  10                  15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr
                  20                  25                  30

Asn Tyr Gly Ile Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
                  35                  40                  45

Glu Trp Val Gly Trp Ile Asn Thr Tyr Thr Gly Glu Pro Thr Tyr
                  50                  55                  60

Ala Ala Asp Phe Lys Arg Arg Phe Thr Phe Ser Leu Asp Thr Ser
                  65                  70                  75

Lys Ser Thr Ala Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
                  80                  85                  90

Thr Ala Val Tyr Tyr Cys Ala Lys Tyr Pro His Tyr Tyr Gly Ser
                  95                 100                 105
```

Ser His Trp Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu
110 115

(2) INFORMATION FOR SEQ ID NO:109:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 110 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:109:

Asp Ile Gln Leu Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
1 5 10 15

Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Asn Glu Gln Leu Ser
20 25 30

Asn Tyr Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
35 40 45

Val Leu Ile Tyr Phe Thr Ser Ser Leu His Ser Gly Val Pro Ser
50 55 60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
65 70 75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
80 85 90

Tyr Ser Thr Val Pro Trp Thr Phe Gly Gln Gly Thr Lys Val Glu
95 100 105

Ile Lys Arg Thr Val
110

(2) INFORMATION FOR SEQ ID NO:110:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 118 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:110:

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
1 5 10 15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Asp Phe Thr
20 25 30

```
His Tyr Gly Met Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
                  35                  40                  45

Glu Trp Val Gly Trp Ile Asn Thr Tyr Thr Gly Glu Pro Thr Tyr
                  50                  55                  60

Ala Ala Asp Phe Lys Arg Arg Phe Thr Phe Ser Leu Asp Thr Ser
                  65                  70                  75

Lys Ser Thr Ala Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
                  80                  85                  90

Thr Ala Val Tyr Tyr Cys Ala Lys Tyr Pro His Tyr Tyr Gly Ser
                  95                 100                 105

Ser His Trp Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu
                 110                 115
```

(2) INFORMATION FOR SEQ ID NO:111:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 110 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:111:

```
Asp Ile Gln Leu Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
  1               5                  10                  15

Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Asn Glu Gln Leu Ser
                  20                  25                  30

Asn Tyr Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
                  35                  40                  45

Val Leu Ile Tyr Phe Thr Ser Ser Leu His Ser Gly Val Pro Ser
                  50                  55                  60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
                  65                  70                  75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
                  80                  85                  90

Tyr Ser Thr Val Pro Trp Thr Phe Gly Gln Gly Thr Lys Val Glu
                  95                 100                 105

Ile Lys Arg Thr Val
                 110
```

(2) INFORMATION FOR SEQ ID NO:112:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 118 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:112:

```
Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
 1               5                  10                  15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr
            20                  25                  30

Asn Tyr Gly Ile Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
            35                  40                  45

Glu Trp Val Gly Trp Ile Asn Thr Tyr Thr Gly Glu Pro Thr Tyr
            50                  55                  60

Ala Ala Asp Phe Lys Arg Arg Phe Thr Phe Ser Leu Asp Thr Ser
            65                  70                  75

Lys Ser Thr Ala Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
            80                  85                  90

Thr Ala Val Tyr Tyr Cys Ala Lys Tyr Pro Tyr Tyr Tyr Gly Thr
            95                 100                 105

Ser His Trp Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu
           110                 115
```

(2) INFORMATION FOR SEQ ID NO:113:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 110 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:113:

```
Asp Ile Gln Leu Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
 1               5                  10                  15

Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Asn Glu Gln Leu Ser
            20                  25                  30

Asn Tyr Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
            35                  40                  45
```

```
Val Leu Ile Tyr Phe Thr Ser Ser Leu His Ser Gly Val Pro Ser
                  50                  55                  60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
                  65                  70                  75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
                  80                  85                  90

Tyr Ser Thr Val Pro Trp Thr Phe Gly Gln Gly Thr Lys Val Glu
                  95                 100                 105

Ile Lys Arg Thr Val
                 110
```

(2) INFORMATION FOR SEQ ID NO:114:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 118 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:114:

```
Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
  1               5                  10                  15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Asp Phe Thr
                  20                  25                  30

His Tyr Gly Met Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
                  35                  40                  45

Glu Trp Val Gly Trp Ile Asn Thr Tyr Thr Gly Glu Pro Thr Tyr
                  50                  55                  60

Ala Ala Asp Phe Lys Arg Arg Phe Thr Phe Ser Leu Asp Thr Ser
                  65                  70                  75

Lys Ser Thr Ala Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
                  80                  85                  90

Thr Ala Val Tyr Tyr Cys Ala Lys Tyr Pro Tyr Tyr Tyr Gly Thr
                  95                 100                 105

Ser His Trp Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu
                 110                 115
```

(2) INFORMATION FOR SEQ ID NO:115:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 110 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:115:

```
Asp Ile Gln Leu Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
 1               5                  10                  15

Gly Asp Arg Val Thr Ile Thr Cys Ser Ala Ser Gln Asp Ile Ser
            20                  25                  30

Asn Tyr Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
            35                  40                  45

Val Leu Ile Tyr Phe Thr Ser Ser Leu His Ser Gly Val Pro Ser
            50                  55                  60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
            65                  70                  75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
            80                  85                  90

Tyr Ser Thr Val Pro Trp Thr Phe Gly Gln Gly Thr Lys Val Glu
            95                 100                 105

Ile Lys Arg Thr Val
           110
```

(2) INFORMATION FOR SEQ ID NO:116:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 118 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:116:

```
Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
 1               5                  10                  15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Asp Phe Thr
            20                  25                  30

His Tyr Gly Met Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
            35                  40                  45

Glu Trp Val Gly Trp Ile Asn Thr Tyr Thr Gly Glu Pro Thr Tyr
            50                  55                  60
```

```
Ala Ala Asp Phe Lys Arg Arg Phe Thr Phe Ser Leu Asp Thr Ser
                  65                  70                  75

Lys Ser Thr Ala Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
                  80                  85                  90

Thr Ala Val Tyr Tyr Cys Ala Lys Tyr Pro Tyr Tyr Tyr Gly Thr
                  95                 100                 105

Ser His Trp Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu
                 110                 115
```

(2) INFORMATION FOR SEQ ID NO:117:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:117:

```
Gly Tyr Xaa Xaa Xaa Xaa Tyr Gly Xaa Asn
  1               5                  10
```

(2) INFORMATION FOR SEQ ID NO:118:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:118:

```
Trp Ile Asn Thr Xaa Thr Gly Glu Pro Thr Tyr Ala Ala Asp Phe
  1               5                  10                  15

Lys Arg
```

(2) INFORMATION FOR SEQ ID NO:119:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:119:

```
Tyr Pro Xaa Tyr Xaa Xaa Xaa Xaa His Trp Tyr Phe Asp Val
  1               5                  10
```

(2) INFORMATION FOR SEQ ID NO:120:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:120:

Xaa Ser Xaa Asp Xaa Xaa Xaa Xaa Thr Xaa
1 5 10

(2) INFORMATION FOR SEQ ID NO:121:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:121:

Xaa Ala Xaa Xaa Xaa Xaa Ser Asn Tyr Leu Asn
1 5 10

(2) INFORMATION FOR SEQ ID NO:122:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:122:

Phe Thr Ser Ser Leu His Ser
1 5

(2) INFORMATION FOR SEQ ID NO:123:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:123:

Gln Gln Tyr Ser Xaa Xaa Pro Trp Thr
1 5

(2) INFORMATION FOR SEQ ID NO:124:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 108 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:124:

```
Asp Ile Gln Xaa Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
 1               5                  10                  15

Gly Asp Arg Val Thr Ile Thr Cys Ser Ala Ser Gln Asp Ile Ser
            20                  25                  30

Asn Tyr Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
            35                  40                  45

Val Leu Ile Tyr Phe Thr Ser Ser Leu His Ser Gly Val Pro Ser
            50                  55                  60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
            65                  70                  75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
            80                  85                  90

Tyr Ser Thr Val Pro Trp Thr Phe Gly Gln Gly Thr Lys Val Glu
            95                 100                 105

Ile Lys Arg
```

(2) INFORMATION FOR SEQ ID NO:125:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 123 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:125:

```
Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
 1               5                  10                  15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Xaa Phe Thr
            20                  25                  30

Xaa Tyr Gly Met Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
            35                  40                  45

Glu Trp Val Gly Trp Ile Asn Thr Tyr Thr Gly Glu Pro Thr Tyr
            50                  55                  60
```

Ala Ala Asp Phe Lys Arg Arg Phe Thr Phe Ser Leu Asp Thr Ser
65 70 75

Lys Ser Thr Ala Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
80 85 90

Thr Ala Val Tyr Tyr Cys Ala Lys Tyr Pro Xaa Tyr Tyr Gly Xaa
95 100 105

Ser His Trp Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu Val Thr
110 115 120

Val Ser Ser

(2) INFORMATION FOR SEQ ID NO:126:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:126:

Gly Tyr Asp Phe Thr His Tyr Gly Met Asn
1 5 10

(2) INFORMATION FOR SEQ ID NO:127:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:127:

Tyr Pro Tyr Tyr Tyr Gly Thr Ser His Trp Tyr Phe Asp Val
1 5 10

(2) INFORMATION FOR SEQ ID NO:128:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:128:

Gly Tyr Xaa Phe Thr Xaa Tyr Gly Met Asn
1 5 10

(2) INFORMATION FOR SEQ ID NO:129:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:129:

```
Tyr Pro Xaa Tyr Tyr Gly Xaa Ser His Trp Tyr Phe Asp Val
 1               5                  10
```

(2) INFORMATION FOR SEQ ID NO:130:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 254 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:130:

```
Met Lys Lys Asn Ile Ala Phe Leu Leu Ala Ser Met Phe Val Phe
-23         -20                 -15                 -10

Ser Ile Ala Thr Asn Ala Tyr Ala Glu Val Gln Leu Val Glu Ser
             -5                   1                   5

Gly Gly Gly Leu Val Gln Pro Gly Gly Ser Leu Arg Leu Ser Cys
         10                  15                  20

Ala Ala Ser Gly Tyr Thr Phe Thr Asn Tyr Gly Met Asn Trp Ile
         25                  30                  35

Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val Gly Trp Ile Asn
         40                  45                  50

Thr Tyr Thr Gly Glu Pro Thr Tyr Ala Ala Asp Phe Lys Arg Arg
         55                  60                  65

Phe Thr Ile Ser Ala Asp Thr Ser Ser Asn Thr Val Tyr Leu Gln
         70                  75                  80

Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys Ala
         85                  90                  95

Lys Tyr Pro His Tyr Tyr Gly Ser Ser His Trp Tyr Phe Asp Val
        100                 105                 110

Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys
        115                 120                 125
```

```
Gly Pro Ser Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser
        130                 135                 140

Gly Gly Thr Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro
        145                 150                 155

Glu Pro Val Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly
        160                 165                 170

Val His Thr Phe Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser
        175                 180                 185

Leu Ser Ser Val Val Thr Val Pro Ser Ser Ser Leu Gly Thr Gln
        190                 195                 200

Thr Tyr Ile Cys Asn Val Asn His Lys Pro Ser Asn Thr Lys Val
        205                 210                 215

Asp Lys Lys Val Glu Pro Lys Ser Cys Asp Lys Thr His Leu
        220                 225                 230
```

(2) INFORMATION FOR SEQ ID NO:131:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 158 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:131:

```
Ser Gly Gly Gly Ser Gly Ser Gly Asp Phe Asp Tyr Glu Lys Met
  1               5                  10                  15

Ala Asn Ala Asn Lys Gly Ala Met Thr Glu Asn Ala Asp Glu Asn
                20                  25                  30

Ala Leu Gln Ser Asp Ala Lys Gly Lys Leu Asp Ser Val Ala Thr
                35                  40                  45

Asp Tyr Gly Ala Ala Ile Asp Gly Phe Ile Gly Asp Val Ser Gly
                50                  55                  60

Leu Ala Asn Gly Asn Gly Ala Thr Gly Asp Phe Ala Gly Ser Asn
                65                  70                  75

Ser Gln Met Ala Gln Val Gly Asp Gly Asp Asn Ser Pro Leu Met
                80                  85                  90

Asn Asn Phe Arg Gln Tyr Leu Pro Ser Leu Pro Gln Ser Val Glu
                95                 100                 105
```

```
Cys Arg Pro Phe Val Phe Ser Ala Gly Lys Pro Tyr Glu Phe Ser
                110                 115                 120

Ile Asp Cys Asp Lys Ile Asn Leu Phe Arg Gly Val Phe Ala Phe
                125                 130                 135

Leu Leu Tyr Val Ala Thr Phe Met Tyr Val Phe Ser Thr Phe Ala
                140                 145                 150

Asn Ile Leu Arg Asn Lys Glu Ser
                155
```

Patent Docket P1093P1D1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Manuel Baca et al. Serial No.: 09/723,752 Filed: November 27, 2000 For: ANTI-VEGF ANTIBODIES	Group Art Unit: 1642 Examiner: Helms, Larry Ronald **CERTIFICATE OF MAILING** I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Assistant Commissioner of Patents, Washington, D.C. 20231 on October 28, 2002 Eileen Ly

CERTIFICATE RE: SEQUENCE LISTING

RESPONSE UNDER 37 CFR § 1.821(f) and (g)

Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

I hereby state that the Sequence Listing submitted herewith is submitted in paper copy and a computer-readable diskette, and that the information recorded in computer readable form is identical to the written sequence listing. I further state that this submission includes no new matter.

Respectfully submitted,

GENENTECH, INC.

Date: October 28, 2002

By: _____
Steven X. Cui
Reg. No. 44,637
Telephone No. (650) 225-8674

09157
PATENT TRADEMARK OFFICE

#123487

1642 (Box Seq)

Patent Docket P1093P1D1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Manuel Baca et al. Serial No.: 09/723,752 Filed: November 27, 2000 For: ANTI-VEGF ANTIBODIES	Group Art Unit: 1642 Examiner: Helms, Larry Ronald **CERTIFICATE OF MAILING** I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Assistant Commissioner of Patents, Washington, D.C. 20231 on October 28, 2002 Eileen Ly

TRANSMITTAL LETTER

Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

Transmitted herewith are the following documents:

1. Amendment and Response to Restriction Requirement;
2. Petition and Fee for One Month Extension of Time (duplicate);
3. Certificate re: Sequence Listing;
4. Sequence Listing and Diskette; and
5. Return Postcard

In the event any additional fees are due in connection with the filing of these documents, the Commissioner is authorized to charge such fees to our Deposit Account No. 07-0630.

Respectfully submitted,

GENENTECH, INC.

Date: October 28, 2002

By: _____
Steven X. Cui
Reg. No. 44,637
Telephone No. (650) 225-8674

09157
PATENT TRADEMARK OFFICE

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/723,752	11/27/2000	Manuel Baca	P1093P1D1	6340

9157 7590 01/17/2003

GENENTECH, INC.
1 DNA WAY
SOUTH SAN FRANCISCO, CA 94080

EXAMINER
HELMS, LARRY RONALD

ART UNIT	PAPER NUMBER
1642	14

DATE MAILED: 01/17/2003

Please find below and/or attached an Office communication concerning this application or proceeding.

PTO-90C (Rev. 07-01)

Office Action Summary	Application No.	Applicant(s)	
	09/723,752	BACA ET AL.	
	Examiner	Art Unit	
	Larry R. Helms	1642	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133).
- Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *04 November 2002* .

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *43-47 and 49-59* is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *43-47,49,50 and 53-59* is/are rejected.

7)☒ Claim(s) *51 and 52* is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☒ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

11)☐ The proposed drawing correction filed on _____ is: a)☐ approved b)☐ disapproved by the Examiner.

If approved, corrected drawings are required in reply to this Office action.

12)☐ The oath or declaration is objected to by the Examiner.

Priority under 35 U.S.C. §§ 119 and 120

13)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____ .

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

14)☐ Acknowledgment is made of a claim for domestic priority under 35 U.S.C. § 119(e) (to a provisional application).

a) ☐ The translation of the foreign language provisional application has been received.

15)☒ Acknowledgment is made of a claim for domestic priority under 35 U.S.C. §§ 120 and/or 121.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO-1449) Paper No(s) _____ .
4) ☐ Interview Summary (PTO-413) Paper No(s). _____ .
5) ☐ Notice of Informal Patent Application (PTO-152)
6) ☒ Other: *See Continuation Sheet* .

Continuation of Attachment(s) 6). Other: notice to comply with sequence requirements.

DETAILED ACTION

Election/Restrictions

1. Upon reconsideration the species election requirement in paper #11 is vacated.

2. Claim 48 has been canceled and claims 47, 49-52 have been amended.

3. Claims 43-47, 49-59 are pending and under examination.

Sequence Requirements

4. It is noted that this application is in sequence compliance, however, the disc containing the substitute sequence listing submitted 10/28/02 was damaged as noted in the CRF Error report supplied with this Office Action. Although the CRF was damaged, the previously submitted CRF was used to search the SEQ ID Nos recited in the claims because those SEQ ID Nos were not altered as stated in the response filed 10/28/02 (see page 3). It is requested that a new CRF be supplied with the response to this Office Action as indicated on the Notice to Comply form enclosed with this Office Action.

Claim Objections

5. Claim 51 is objected to because of the following informalities: Claim 51 contains a typographical error in that SEQ ID NO: 115 is for a light chain not a heavy chain and SEQ ID NO: 116 is the sequence for a heavy chain. The claim will be interpreted to be a light chain of SEQ ID NO:115 and a heavy chain of SEQ ID NO:116. Appropriate correction is required.

Specification

6. The disclosure is objected to because of the following informalities:

a. The first line of the specification should be updated to indicate 08/833,504 is no a provisional application 60/126,446.

Appropriate correction is required.

Claim Rejections - 35 USC § 112

7. The following is a quotation of the first paragraph of 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

8. Claims 47, 49-50 are rejected under 35 U.S.C. 112, first paragraph, because the specification, while being enabling for a method of inhibiting VEGF-induced angiogenesis in a subject by administration of an antibody comprising a light chain with CDRL1 of SEQ ID NO:4, CDRL2 of SEQ ID NO:5, CDRL3 of SEQ ID NO:6 and a heavy chain with CDRH1 of SEQ ID NO:128 or 1, CDRH2 of SEQ ID NO:2, and CDRH3 of SEQ ID NO:129 or 3, does not reasonably provide enablement for a method of inhibiting VEGF-induced angiogenesis in a subject by administration of an antibody with the specified light chain sequence as recited in the claims and any heavy chain or an antibody with the CDRs specified in the claims and any light chain. The specification

does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to use the invention commensurate in scope with these claims.

Factors to be considered in determining whether undue experimentation is required, are summarized in Ex parte Forman, 230 USPQ 546 (BPAI 1986). They include the nature of the invention, the state of the prior art, the relative skill of those in the art, the amount of direction or guidance disclosed in the specification, the presence or absence of working examples, the predictability or unpredictability of the art, the breadth of the claims, and the quantity of experimentation which would be required in order to practice the invention as claimed.

The claims are broadly drawn to a method of inhibiting VEGF-induced angiogenesis with an antibody with specific CDRs of a light chain and any CDRs of a heavy chain or an antibody with specific CDRs from a heavy chain and any CDRs from any light chain. The specification teaches a method with specific antibodies with specific CDRs from a light chain and a heavy chain (see figures 1A-1B). the specification does not enable an antibody as broadly claimed in a method of inhibiting angiogenesis.

It is well established in the art that the formation of an intact antigen-binding site generally requires the association of the complete heavy and light chain variable regions of a given antibody, each of which consists of three CDRs which provide the majority of the contact residues for the binding of the antibody to its target epitope. The amino acid sequences and conformations of each of the heavy and light chain CDRs are critical in maintaining the antigen binding specificity and affinity which is

characteristic of the parent immunoglobulin. It is expected that all of the heavy and light chain CDRs in their proper order and in the context of framework sequences which maintain their required conformation, are required in order to produce a protein having antigen-binding function and that proper association of heavy and light chain variable regions is required in order to form functional antigen binding sites. Even minor changes in the amino acid sequences of the heavy and light variable regions, particularly in the CDRs, may dramatically affect antigen-binding function as evidenced by Rudikoff et al (Proc Natl Acad Sci USA 1982 Vol 79 page 1979). Rudikoff et al. teach that the alteration of a single amino acid in the CDR of a phosphocholine-binding myeloma protein resulted in the loss of antigen-binding function. It is unlikely that antibodies as defined by the claims which may contain less than the full complement of CDRs from the heavy and light chain variable regions have the required binding function or can be used in the claimed method. The specification provides no direction or guidance regarding how to produce antibodies as broadly defined by the claims. Undue experimentation would be required to produce the invention commensurate with the scope of the claims from the written disclosure alone.

Therefore, in view of the lack of guidance in the specification and in view of the discussion above one of skill in the art would be required to perform undue experimentation in order to practice the claimed invention.

Priority

9. The instant application claims priority to provisional application 60/126,446, filed 4/7/97. Claims 43, recites the limitation of "a Kd value of no more than about 1 X 10^{-8}M" and claim 46 recites the limitation of "in an A673 in vivo tumor model". The limitations have support in the instant application, however, it appears that there is not support for these limitations in the 60/126,446 application. As such the priority date granted to claims 43-47, 49-59 is 8/6/97.

Claim Rejections - 35 USC § 103

10. The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

The factual inquiries set forth in *Graham* **v.** *John Deere Co.*, 383 U.S. 1, 148 USPQ 459 (1966), that are applied for establishing a background for determining obviousness under 35 U.S.C. 103(a) are summarized as follows:

1. Determining the scope and contents of the prior art.
2. Ascertaining the differences between the prior art and the claims at issue.
3. Resolving the level of ordinary skill in the pertinent art.
4. Considering objective evidence present in the application indicating obviousness or nonobviousness.

This application currently names joint inventors. In considering patentability of the claims under 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of 35 U.S.C. 103(c) and potential 35 U.S.C. 102(e), (f) or (g) prior art under 35 U.S.C. 103(a).

11. Claims 43-47, 49-50, 53-56 are rejected under 35 U.S.C. 103(a) as being unpatentable over Ferrara et al (WO 94/10202, published 5/11/94) and further in view of Adair et al (WO 91/09967, published 7/11/91) and Yelton et al (The Journal of Immunology 155:1994-2004, 1995) and as evidenced from the specification.

The claims recite a method of inhibiting VEGF-induced angiogenesis in a subject by administration of an antibody wherein the subject has a tumor wherein the antibody binds no more than 10-9M, and 5mg/kg inhibits at least 50% of tumor growth in a A673 in vivo model, the antibody comprises CDRs recited in the claims, the antibody is a full length antibody, a IgG, and a Fab.

Ferrara et al teach an anti-VEGF antibody (see abstract). Ferrara et al also teach a humanized antibody (see page 8, lines 13-31) and the effect of the antibodies in tumor cell growth and angiogenesis (see page 23-24 and page 4). Ferrara et al also teach methods of inhibiting VEGF-induced angiogenesis in a subject and the subject can have cancer and the antibody was tested in a A673 model (see abstract and

Example 2) and the humanized antibody binds with 10-9M affinity (see page 8). Ferrara et al does not teach a specific method for humanization or obtaining the CDR sequence of the antibody. This deficiency is made up for in the teachings of Adair et al and Yelton et al.

Adair et al teach a method of antibody humainzation by CDR grafting and framework modifications and methods of obtaining the amino acid sequences of antibodies from hybridomas and fragments of the antibody such as Fabs (see abstract and entire document).

Yelton et al teach an affinity maturation method comprising alterations in the CDRs of the heavy chain (see abstract).

It would have been prima facie obvious to one of ordinary skill in the art at the time the claimed invention was made to have produced a method of inhibiting VEGF-induced angiogenesis in a subject with cancer by administration of a humanized antibody of Ferrara humanized by the methods of Adair et al and Yelton et al.

One of ordinary skill in the art would have been motivated to and had a reasonable expectation of success to produce the claimed method with a humanized anti-VEGF antibody because "most Mabs are of rodent origin, they are naturally antigenic in humans and thus can give rise to an undesirable immune response termed the HAMA" (see page 2). In addition, one of ordinary skill in the art would have been motivated and had a reasonable expectation of success to produce the claimed method because Ferrara et al teach the antibody can be humanized and the tumors from A4.6.1 treated animals were smaller than those tumors in mice treated with a control antibody

(see Figure 5). In addition, one of ordinary skill in the art would have been motivated to and had a reasonable expectation of success to produce the claimed method because Yelton et al teach a method for affinity maturation of an antibody in order to "change the form, affinity, and potentially the specificity of Abs to optimize them for delivering a wide variety of therapeutic agents to tumor cells." (See page 2002 last paragraph)

Moreover, it would have been obvious to humanize the A4.6.1 antibody of Ferrara et al by the methods of Adair et al and Yelton et al because Ferrara et al teach human VEGF and in view of Adair and Yelton et al it would be obvious to humanize the antibody for therapy for inhibiting VEGf-induced angiogenesis.

As evidenced from the specification the A4.6.1 antibody of Ferrara et al has the CDRs as recited in claims 47, 49-50 (see Figure 1A and 1B of the specification).

It is the Examiner's position that the antibody produced by humanizing Ferrara et al's antibody with Adair et al's and Yelton et al's method would produce a humanized antibody that would have the binding and inhibition characteristics claimed in the claimed method. One of ordinary skill in the art would reasonably conclude that Ferrara et al's antibody humanized with Adair et al's and Yelton et al's method also possesses (1) the same binding affinity to the human VEGF, and (2) inhibits angiogenesis and tumor growth of at least about 50% in A673 in vivo tumor model, therefore, it appears that Ferrara et al's antibody humanized with Adair et al's and Yelton et al's method would produce a humanized antibody that is identical to the claimed antibody. Since the Patent and Trademark Office does not have the facilities for examining and comparing the claimed humanized antibody with the humanized antibody of Ferrara et

al's antibody humanized with Adair et al's and Yelton et al's method, the burden of proof is upon the Applicants to show an unobvious distinction between the structural and functional characteristics of the claimed antibody and the antibody of the prior art. See In re Best, 562 F.2d 1252, 195 U.S.P.Q. 430 (CCPA 197) and Ex parte Gray, 10 USPQ 2d 1922 1923 (PTO Bd. Pat. App. & Int.).

Therefore, the invention as a whole was prima facie obvious to one of ordinary skill in the art at the time the invention was made, as evidenced by the references.

12. Claims 43-47, 49-50, 53-59 are rejected under 35 U.S.C. 103(a) as being unpatentable over Ferrara et al (WO 94/10202, published 5/11/94) and further in view of Adair et al (WO 91/09967, published 7/11/91) and Yelton et al (The Journal of Immunology 155:1994-2004, 1995) and as evidenced from the specification as applied to claims 43-47, 49-50, 53-56 above, and further in view of Lopez et al (Invest Opthal. And Visual Science 37:855, 4/96).

Claims 43-47, 49-50 and 53-56 have been described supra. Claims 57-59 recite wherein the subject has age related macular degeneration and the antibody is administered at a dose of at least about 0.5 mg/kg.

Ferrara et al has been described supra. Ferrara et al also teach administration at 0.1 to 100 mg/kg (see page 15). Ferrara et al does not teach a humanized antibody by administration to a subject with age related macular degeneration. This deficiency is made up for in the teachings of Lopez et al.

Adair et al and Yelton et al have been described supra.

Lopez et al teach VEGF may be important in the progression of ARMD (see page 865) and VEGF is a critical factor in CNVM development (see page 856).
It would have been prima facie obvious to one of ordinary skill in the art at the time the claimed invention was made to have produced a method of inhibiting VEGF-induced angiogenesis in a subject with cancer by administration of a humanized antibody of Ferrara humanized by the methods of Adair et al and Yelton et al.

It would have been prima facie obvious to one of ordinary skill in the art at the time the claimed invention was made to have produced a method of inhibiting VEGF-induced angiogenesis in a subject with AMD by administration of a humanized antibody of Ferrara humanized by the methods of Adair et al and Yelton et al in view of Lopez et al.

One of ordinary skill in the art would have been motivated to and had a reasonable expectation of success to produce the claimed method because Ferrera et al teach the methods for inhibition of angiogenesis in a subject with many diseases and in view of Lopez which teaches VEGF is involved in angiogenesis in ARMD it would be obvious to inhibit ARMD with a humanized antibody to VEGF.

Therefore, the invention as a whole was prima facie obvious to one of ordinary skill in the art at the time the invention was made, as evidenced by the references.

Conclusion

13. No Claims are allowed. Claims 51 and 52 objected to as being dependent upon a rejected base claim, but would be allowable if rewritten in independent form including all of the limitations of the base claim and any intervening claims.

14. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Larry R. Helms, Ph.D, whose telephone number is (703) 306-5879. The examiner can normally be reached on Monday through Friday from 7:00 am to 4:30 pm, with alternate Fridays off. If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Anthony Caputa, can be reached on (703) 308-3995. Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to the Group receptionist whose telephone number is (703) 308-0196.

14. Papers related to this application may be submitted to Group 1600 by facsimile transmission. Papers should be faxed to Group 1600 via the PTO Fax Center located in Crystal Mall 1. The faxing of such papers must conform with the notice published in the Official Gazette, 1096 OG 30 (November 15, 1989). The CM1 Fax Center telephone number is (703) 308-4242.

Respectfully,

Larry R. Helms Ph.D.

703-306-5879

Application No. 09/723752

NOTICE TO COMPLY WITH REQUIREMENTS FOR PATENT APPLICATIONS CONTAINING NUCLEOTIDE SEQUENCE AND/OR AMINO ACID SEQUENCE DISCLOSURES

The nucleotide and/or amino acid sequence disclosure contained in this application does not comply with the requirements for such a disclosure as set forth in 37 C.F.R. 1.821 - 1.825 for the following reason(s):

☐ 1. This application clearly fails to comply with the requirements of 37 C.F.R. 1.821-1.825. Applicant's attention is directed to these regulations, published at 1114 OG 29, May 15, 1990 and at 55 FR 18230, May 1, 1990.

☐ 2. This application does not contain, as a separate part of the disclosure on paper copy, a "Sequence Listing" as required by 37 C.F.R. 1.821(c).

☐ 3. A copy of the "Sequence Listing" in computer readable form has not been submitted as required by 37 C.F.R. 1.821(e).

☐ 4. A copy of the "Sequence Listing" in computer readable form has been submitted. However, the content of the computer readable form does not comply with the requirements of 37 C.F.R. 1.822 and/or 1.823, as indicated on the attached copy of the marked -up "Raw Sequence Listing."

☒ 5. The computer readable form that has been filed with this application has been found to be damaged and/or unreadable as indicated on the attached CRF Diskette Problem Report. A Substitute computer readable form must be submitted as required by 37 C.F.R. 1.825(d).

☐ 6. The paper copy of the "Sequence Listing" is not the same as the computer readable from of the "Sequence Listing" as required by 37 C.F.R. 1.821(e).

☐ 7. Other: _____

Applicant Must Provide:

☒ An initial or substitute computer readable form (CRF) copy of the "Sequence Listing".

☐ An initial or substitute paper copy of the "Sequence Listing", as well as an amendment directing its entry into the specification.

☒ A statement that the content of the paper and computer readable copies are the same and, where applicable, include no new matter, as required by 37 C.F.R. 1.821(e) or 1.821(f) or 1.821(g) or 1.825(b) or 1.825(d).

For questions regarding compliance to these requirements, please contact:

For Rules Interpretation, call (703) 308-4216
For CRF Submission Help, call (703) 308-4212
For PatentIn software help, call (703) 308-6856

PLEASE RETURN A COPY OF THIS NOTICE WITH YOUR RESPONSE

Notice of References Cited	Application/Control No. 09/723,752	Applicant(s)/Patent Under Reexamination BACA ET AL.	
	Examiner Larry R. Helms	Art Unit 1642	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
	A	US-			
	B	US-			
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N	WO 94/10202	05-1994	World	Ferrara et al	-----
	O	WO 91/09967	07-1991	World	Adair et al	-----
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Lopez et al Invest. Opthal. and Visual Science 37:855, 1996
	V	Rudikoff et al., PNAS 79:1979, 1982
	W	Yelton et al., J. of Immunol 155:1994-2004, 1995
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Patent Docket P1093P1D1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Manuel Baca et al. Serial No.: 09/723,752 Filed: November 27, 2000 For: ANTI-VEGF ANTIBODIES	Group Art Unit: 1642 Examiner: Helms, Larry Ronald EXPRESS MAIL LABEL NO.: EV 351 926 729 US DATE OF DEPOSIT: JULY 17, 2003

AMENDMENT TRANSMITTAL

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Transmitted herewith is an amendment in the above-identified application.

The fee has been calculated as shown below.

	Claims Remaining After Amendment		Highest No. Previously Paid For	Present Extra	Rate	Additional Fees
Total	17	-	20	0	18	$0.00
Independent	3	-	3	0	84	$0.00
__ 0Multiple dependent claim(s), if any					280	$0.00
					Total Fee Calculation	$0.00

__X__ No additional fee is required.
_____ The Commissioner is hereby authorized to charge Deposit Account No. 07-0630 in the amount of $. **A duplicate copy of this transmittal is enclosed.**
__X__ Petition for Extension of Time is enclosed.

The Commissioner is hereby authorized to charge any additional fees required under 37 CFR 1.16 and 1.17, or credit overpayment to Deposit Account No. 07-0630. **A duplicate copy of this sheet is enclosed.**

Respectfully submitted,
GENENTECH, INC.

Date: July 17, 2003

By: Steven X. Cui
Steven X. Cui
Reg. No. 44,637
Telephone No. (650) 225-8674

09157
PATENT TRADEMARK OFFICE

Patent Docket P1093P1D1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Manuel Baca et al. Serial No.: 09/723,752 Filed: November 27, 2000 For: ANTI-VEGF ANTIBODIES	Group Art Unit: 1642 Examiner: Helms, Larry Ronald EXPRESS MAIL LABEL NO.: EV 351 926 729 US DATE OF DEPOSIT: JULY 17, 2003

CERTIFICATE RE: SEQUENCE LISTING

RESPONSE UNDER 37 CFR § 1.825(d)

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

In response to the Notice to Comply form accompanied with the Office Action dated January 17, 2003, a Sequence Listing in a computer-readable diskette is submitted herewith pursuant to 37 CFR 1.825(d), to replace the previously submitted computer-readable diskette that was found to be damaged by the Office. I hereby state that the information recorded in the computer readable form is identical to the written sequence listing submitted previously on October 28, 2002. I further state that this submission includes no new matter.

Respectfully submitted,

GENENTECH, INC.

Date: July 17, 2003

By: _____
Steven X. Cui
Reg. No. 44,637
Telephone No. (650) 225-8674

09157
PATENT TRADEMARK OFFICE

EXPRESS MAIL LABEL NO.: EV 351 926 729 US
DATE OF DEPOSIT: JULY 17, 2003

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Appl. No.:	09/723,752	Attorney Docket No.	P1093P1D1
Applicant:	Manuel Baca et al.	TC/A.U.:	1744
Filed:	November 27, 2000	Examiner:	Helms, Larry Ronald
Title:	ANTI-VEGF ANTIBODIES	Confirmation No.	6340
		Customer No.	09157

Commissioner for Patents
P.O. Box 1450
Alexandria VA 22313-1450

RESPONSE TO NON FINAL OFFICE ACTION UNDER 37 C.F.R. § 1.111

Sir:

This document is responsive to the Office Action mailed January 17, 2003 (Paper No. 14) for which a three month period for response was given. This response is timely filed with a Petition and fees for Three-Month Extension of Time. In view of the amendments and remarks provided herein below, reconsideration and allowance are respectfully requested.

Amendments to the Specification begin on page 2 of this paper.
Amendments to the Claims are reflected in the listing of claims which begins on page 3 of this paper.
Remarks begin on page 6 of this paper.
An **Appendix** including Clean Set of All Pending Claims is attached following page 11 of this paper.

Amendments to the Specification:

Please replace the paragraph beginning at page 1, line 9 with the following amended paragraph:

This application is a divisional of the co-pending U.S. Application Serial No. 08/908,469, filed August 6, 1997, which claims priority under 35 USC 119 to the provisional U.S. Application Serial No. 60/126,446, filed April 7, 1997, which applications are incorporated herein by reference.

Amendments to the Claims:

This listing of claims will replace all prior versions, and listings, of claims in the application:

Listing of Claims:

43. (Currently Amended) A method for inhibiting VEGF-induced angiogenesis in a subject, comprising administering to said subject an effective amount of a humanized anti-VEGF antibody which (a) binds human VEGF with a K_d value of no more than about 1 x 10^{-8}M; (b) has an ED50 value of no more than about 5nM for inhibiting VEGF-induced proliferation of endothelial cells *in vitro*; and (c) inhibits VEGF-induced angiogenesis *in vivo*, wherein 5mg/kg of said humanized antibody inhibits at least about 50% of tumor growth in an A673 *in vivo* tumor model.

44. (Currently Amended) The method of claim 43, wherein said humanized anti-VEGF antibody ~~which~~ binds human VEGF with a K_d value of no more than about 1 x 10^{-9}M.

45. (Previously presented) The method of claim 43, wherein said subject has a tumor.

46. (Currently Amended) The method of claim 45, wherein 5mg/kg of said humanized antibody inhibits at least about ~~50~~80% of tumor growth in an A673 *in vivo* tumor model.

47. (Currently Amended) The method of claim 43, said humanized anti-VEGF antibody having a heavy chain and a light chain, wherein the heavy chain variable domain comprises four framework regions (FR) and three complementarity determining regions (CDR) as a contiguous sequence represented by the formula: FR1-CDRH1-FR2-CDRH2-FR3-CDRH3-FR4, wherein the four FRs are derived from a consensus human antibody heavy chain framework region sequence and the three CDRs are derived from a non-human anti-VEGF antibody, and wherein the light chain variable domain comprises four FRs and three CDRs as a contiguous sequence represented by the formula: FR1-CDRL1-FR2-CDRL2-FR3-CDRL3-FR4, wherein the four FRs are derived from a consensus human antibody light chain framework region sequence and the three CDRs are derived from the non-human anti-VEGF antibody~~comprises a variable domain comprising the following complementarity determining region (CDR) amino acid sequences: CDRH1 (GYX_1FTX_2YGMN, wherein X_1 is T or D and X_2 is N or H; SEQ ID NO: 128), CDRH2 (WINTYTGEPTYAADFKR; SEQ ID NO:2) and CDRH3 ($YPX_1YYGX_2SHWYFDV$, wherein X_1~~

~~is Y or H and X_2 is S or T; SEQ ID NO: 129)~~.

49. (Currently Amended) The method of claim 47, wherein the heavy chain ~~comprises a~~ variable domain ~~comprising~~ comprises the following CDR amino acid sequences: CDRH1 (GYX_1FTX_2YGMN, wherein X_1 is T or D and X_2 is N or H; SEQ ID NO: 128), CDRH2 (WINTYTGEPTYAADFKR; SEQ ID NO:2) and CDRH3 ($YPX_1YYGX_2SHWYFDV$, wherein X_1 is Y or H and X_2 is S or T; SEQ ID NO: 129);~~CDR amino acid sequences: CDRH 1 (GYTFTNYGMN; SEQ ID NO: 1), CDRH2 (WINTYTGEPTYAADFKR; SEQ ID NO:2) and CDRH3 (YPHYYGSSHWYFDV; SEQ ID NO:3)~~ and wherein the light chain variable domain comprises the following CDR amino acid sequences: CDRL1 (SASQDISNYLN; SEQ ID NO:4), CDRL2 (FTSSLHS SEQ ID NO:5) and CDRL3 (QQYSTVPWT; SEQ ID NO:6).

50. (Currently Amended) The method of claim 47, wherein the heavy chain variable domain comprises the following CDR amino acid sequences: CDRH1 (GYTFTNYGMN; SEQ ID NO:1), CDRH2 (WINTYTGEPTYAADFKR; SEQ ID NO:2) and CDRH3 (YPHYYGSSHWYFDV; SEQ ID NO:3);~~wherein~~ and wherein the light chain ~~of the humanized anti-VEGF antibody comprises a~~ variable domain ~~comprising~~ comprises the following CDR amino acid sequences: CDRL1 (SASQDISNYLN; SEQ ID NO:4), CDRL2 (FTSSLHS SEQ ID NO:5) and CDRL3 (QQYSTVPWT; SEQ ID NO:6).

51. (Currently Amended) A method for inhibiting VEGF-induced angiogenesis in a subject, comprising administering to said subject an effective amount of a humanized anti-VEGF antibody which binds human VEGF with a K_d value of no more than about 1×10^{-8}M~~The method of claim 43~~, said humanized anti-VEGF antibody comprising a heavy chain variable domain sequence of SEQ ID NO:116 and a light chain variable domain sequence of SEQ ID NO:115.

52. (Currently Amended) A method for inhibiting VEGF-induced angiogenesis in a subject, comprising administering to said subject an effective amount of a humanized anti-VEGF antibody which binds human VEGF with a K_d value of no more than about 1×10^{-8}M~~The method of claim 43~~, said humanized anti-VEGF antibody comprising a heavy chain variable domain sequence of SEQ ID NO:7 and a light chain variable domain sequence of SEQ ID NO:8.

53. (Previously presented) The method of claim 43, wherein said humanized anti-VEGF

antibody is a full length antibody.

54. (Previously presented) The method of claim 53, wherein said humanized anti-VEGF antibody is a human IgG.

55. (Previously presented) The method of claim 43, wherein said humanized anti-VEGF antibody is an antibody fragment.

56. (Previously presented) The method of claim 55, wherein said humanized anti-VEGF antibody is a Fab.

57. (Previously presented) The method of claim 43, wherein said subject has a retinal disease.

58. (Previously presented) The method of claim 57, wherein said retinal disease is age-related macular degeneration (AMD).

59. (Previously presented) The method of claim 58, wherein the humanized anti-VEGF antibody is administered to the subject at a dose of at least about 0.5mg/kg.

60. (New) The method of claim 47, wherein the heavy chain variable domain FR has at least one substitution wherein the human FR residue is replaced by a corresponding residue from the non-human anti-VEGF antibody, said residue is selected from the following positions: 37H, 49H, 67H, 69H, 71H, 73H, 75H, 76H, 78H and 94H; and wherein the light chain variable domain FR has at least one substitution wherein the human FR residue is replaced by a corresponding residue from the non-human anti-VEGF antibody, said residue is selected from the following positions: 4L, 46L and 71L (positions according to Kabat numbering).

REMARKS

Formal Matters

Claims 43-47 and 49-60 are pending in the application. Claims 43,44,46,47,49-52 are amended and new claim 60 is added. The amendments are fully supported by the specification as filed, and accordingly, do not introduce new matter. Examples of support for each of the amended or new claims are found at the following locations in the specification:

Claim 43: page 3, lines 2-8; claims as originally filed;

Claim 46: page 20, lines 13-15;

Claim 47: page 21, lines 12; page 22, lines 19-29;

Claim 49: page 3, lines 9-13; page 4, lines 2-4; claims as originally filed;

Claim 50: page 3, lines 13-16; page 4, lines 2-4; claims as originally filed;

Claim 51: claims as originally filed;

Claim 52: claims as originally filed;

Claim 60: page 21, lines 13-23; page 22, line 30-page 23, line 6.

The specification has been amended to update the priority information. And a new CRF is submitted to replace the damaged one, pursuant to the Notice to Comply form accompanied with the Office Action.

Claim Objections

Claim 51 is objected to because of a typographical error in identifying the corresponding light and heavy chain sequences recited in the claim. Applicants submit that the claim was amended to correct the error in a Supplemental Amendment submitted January 9, 2003.

Rejections Under 35 U.S.C. § 112, First Paragraph

Claims 47, 49-50 are rejected under 35 U.S.C. § 112, first paragraph because the specification allegedly does not enable an antibody as broadly claimed in a method of inhibiting angiogenesis. According to the Examiner, the claims are broadly drawn to a method of inhibiting VEGF-induced angiogenesis with an antibody with specific CDRs of a light chain and any CDRs of a heavy chain, or an antibody with specific CDRs from a heavy chain and any CDRs from any light chain (emphasis added by the applicants). Meanwhile, the specification allegedly provides no direction or guidance regarding how to produce antibodies as broadly defined by the claims, which may contain less than the full complement of CDRs from the heavy and light chain

variable regions. Furthermore, according to the Examiner, it is unlikely that such antibodies have the required binding function or can be used in the claimed methods, because it is expected that all of the heavy and light chain CDRs in their proper order and in the context of framework sequences which maintain their required conformation, are required in order to produce a protein having antigen-binding function.

Applicants submit that claim 47 has been amended to further clarify that the humanized anti-VEGF antibody used in the claimed method comprises a heavy chain and a light chain, wherein the heavy chain variable domain comprises four FRs and three CDRs as a contiguous sequence represented by the formula: FR1-CDRH1-FR2-CDRH2-FR3-CDRH3-FR4, wherein the four FRs are derived from a consensus human antibody heavy chain framework region sequence and the three CDRs are derived from a non-human anti-VEGF antibody, and wherein the light chain variable domain comprises four FRs and three CDRs as a contiguous sequence represented by the formula: FR1-CDRL1-FR2-CDRL2-FR3-CDRL3-FR4, wherein the four FRs are derived from a consensus human antibody light chain framework region sequence and the three CDRs are derived from the non-human anti-VEGF antibody. Thus, the amended claim 47 is directed to a humanized anti-VEGF antibody with heavy and light chain variable domains in a sequence formula that is well known as sufficient to provide intact antigen-binding. Furthermore, all the CDRs are derived from the same non-human anti-VEGF antibody, thus maintaining the human VEGF binding specificity in the resulting humanized antibody. Claims 49-50 further recite specific CDR sequences for both the light and heavy chain variable domains of the humanized anti-VEGF antibody.

With regard to the Examiner's concern, citing Rudikoff et al., that minor changes in the variable domains, particularly in the CDRs, may dramatically affect antigen-binding function, Applicants submit that the present application provides adequate teachings with ample working examples as to how to make amino acid changes in the variable domains in order to obtain humanized anti-VEGF antibodies with desirable antigen binding affinity and biological activities. For example, Specification at pages 21-23 teaches that the FRs of the humanized antibody which is derived from human FRs may preferably contain residue substitutions wherein the human FR residue(s) is replaced by corresponding non-human residue or a totally different residue, and the resulting antibodies can be subject to, for example, phagemid library display for selection of antibodies having desired antigen-binding affinity. Moreover, the specification teaches further making variants of a humanized antibody in order to obtain even stronger binding affinity. See,

for example, pages 27-30. Example 3 on pages 67-80 also describes how to make amino acid substitutions in both FRs and CDRs in order to obtain "affinity matured" variants with higher binding affinities.

Indeed, the non-limiting working examples of the specification provide detailed teachings for the claimed invention. The Examples describe three different methods for making different sets of anti-VEGF antibodies, all having individual humanized antibodies or antibody variants with desirable properties from therapeutic perspectives, as presently claimed. Specifically, Example 1 describes methods and materials that resulted in a series of humanized anti-VEGF F(ab) variants. One of these variants, F(ab)-12, exhibited a K_d value of $1.8x\ 10^{-9}M$ in a VEGF binding assay. Table 3 on page 57. F(ab)-12 was used to construct a full length mAb, rhuMAb VEGF, which also exhibited the desirable properties as claimed. Example 2 describes methods and materials that resulted in a series of humanized Fab variants selected from a humanized A4.6.1 phagemid Fab library. One of the phage selected clones, hu2.10V, exhibited a K_d value of $9.3x10^{-9}M$ in a VEGF binding assay. Table 7 on page 67. Lastly, Example 3 describes using methods of CDR randomization, affinity maturation by monovalent Fab phage display, and cumulative combination of mutations to enhance the affinity of a humanized anti-VEGF antibody. As the result, several antibodies with high binding affinity were created, including Y0313-1, Y0238-3, and Y0317. Table 15 on page 80 and discussion on page 81. All the above exemplified antibodies or antibody variants have distinct sequence structures, yet exhibited similar desirable properties that are encompassed by the present claims.

In view of the amendments and the above remarks, Applicants submit that the claims are in compliance with 35 USC §112, first paragraph, and respectfully request the rejection be reconsidered and withdrawn.

Rejection Under 35 U.S.C. § 103(a)

Claims 43-47, 49-50 and 53-56 are rejected under 35 U.S.C. §103(a) as allegedly being unpatentable over Ferrara et al. WO 94/10202 ("Ferrara et al"), and further in view of Adair et al. WO91/09967 ("Adair et al") and Yelton et al. (1995)*J. Immn.* 155:1994-2004 ("Yelton et al"). Claims 43-47, 49-50 and 53-59 are also rejected under 35 U.S.C. §103(a) as allegedly being unpatentable over Ferrara et al. and further in view of Adair et al. and Yelton et al. as applied to claims 43-47, 49-50, 53-56, and further in view of Lopez et al. (1996) Invest. Opthal. and Visual Sci. 37:855-868 ("Lopez et al") concerning the role of VEGF in the progression of ARMD.

According to the Examiner, it would have been prima facie obvious to one of ordinary skill in the art at the time the claimed invention was made to have produced a method of inhibiting VEGF-induced angiogenesis in a subject with cancer or AMD, by administration of a humanized antibody of Ferrara et al humanized by the methods of Adair et al and Yelton et al. Moreover, the Examiner took the position that the antibody so produced would have the binding and inhibition characteristics claimed in the present invention. Applicants respectfully traverse these rejections.

Claim 43 has been amended to a method for inhibiting VEGF-induced angiogenesis in a subject, comprising administering to said subject an effective amount of a humanized anti-VEGF antibody which (a) binds human VEGF with a K_d value of no more than about 1 x 10^{-8}M; (b) has an ED50 value of no more than about 5nM for inhibiting VEGF-induced proliferation of endothelial cells *in vitro*; and (c) inhibits VEGF-induced angiogenesis *in vivo*, wherein 5mg/kg of said humanized antibody inhibits at least about 50% of tumor growth in an A673 *in vivo* tumor model. Thus, to render the present claims obvious over the cited references, it must be shown that one of ordinary skill in the art would have been motivated and had a reasonable expectation of success to produce a humanized anti-VEGF antibody having all of the above-recited properties. Applicants submit that the teachings of the cited references, even if combined, would not have rendered obvious a humanized anti-VEGF antibody with the desired VEGF binding affinity as presently claimed, much less the additionally claimed potencies both *in vitro* and *in vivo* (i.e., elements (b) and (c) of claim 43).

As disclosed in the specification of the present application, while non-human anti-VEGF neutralizing antibodies capable of suppressing angiogenesis related conditions (including the growth of a variety of human tumor cell lines in nude mice) and uses thereof were known in the art, the present invention is directed to humanized anti-VEGF antibodies with desirable properties from a therapeutic perspective. See specification at page 2, lines 19-29. The invention was the result of a series of experiments employing different approaches for humanizing an anti-VEGF antibody. One of ordinary skill in the art applying the general methods of Adair et al or Yelton et al to the anti-VEGF antibody of Ferrara et al would not have had reasonable expectation of success in producing a humanized anti-VEGF antibody having both the binding affinity and the inhibition potencies as currently claimed.

In particular, applicants point out that it has been known in the art, and even acknowledged in the cited references Adair et al and Yelton et al, that an antibody with high

binding affinity to its antigen does not necessarily exert desired efficacy when used in the context of cultured cells or *in vivo* therapeutic treatment. For example, Adair et al discloses a method of humanization combining CDR grafting with framework residue substitutions, based on studies of an anti-CD3 antibody OKT3. When tested for biological activities, even those modified antibodies with increased antigen binding affinities behaved differently in an unpredictable manner. In Example 5 (pages 61-64), for example, a number of murine anti-TGF-α mAbs were CDR-grafted (and FR residues swapped) according to the protocol used for OKT3 antibodies. Some of the resultant variants showed binding affinities similar to that of the murine or chimeric counterpart antibodies. These variant antibodies were then assessed in an L929 cell competition assay in which the antibody functionally competes against the TNF receptor on L929 cells for binding to TNF in solution. The results showed that while some of the resultant antibodies were able to compete well in the L929 assay, many others failed to effectively compete with and block the TNF receptor-ligand interaction. Specifically, gL221/gH341, the humanized version of 61E71, was approximately 10% as active as murine 61E71 (page 61); the humanized version of hTNF3 bound well to TNF-α, but competed very poorly in the L929 assay (page 63); and the humanized 101.4 antibodies were at least an order of magnitude less able to compete for TNF against the TNF receptor on L929 cells (page 64). Thus, the Adair et al reference itself showed that increased binding affinity of a humanized antibody as a result of using the methods taught therein can not predict improved competitive activity in a cell assay.

Yelton et al describes affinity maturation of a chimeric anti-carcinoma antibody, BR96, by codon-based mutagenesis. BR96 is a mAb recognizing Lewis Y (Le^y)-related antigens expressed on the surface of many human carcinomas. The affinity mutants of BR96 were tested for their binding affinities to either an enzyme conjugate of synthetic Le^y tetrasaccharide (sLe^y) serving as an isolated antigen, or carcinoma cell lines expressing on their surface the Le^y antigen. The results provided by Yelton et al clearly show that the binding affinity to sLe^y does not always correlate with the binding affinity to tumor cells with Le^y expressed and bound on their surface. For example, a mutant clone M4 was shown to bind sLe^y with a 3-4 fold greater reactivity than M1 (another mutant), and an approximately 15-20 fold increase compared with the BR96 parent. Yet it did not show any improvement over M1 in binding to H3396 tumor cell membranes. Pages 1999-2000. Thus, antibody mutants generated according to Yelton et al would not necessarily have desired binding affinity to an antigen in a native state, much less any therapeutic efficacy. Indeed, the authors of the references went on to postulate that increasing the affinity of an

antibody (specific to a tumor antigen) may not bring a therapeutic advantage in treating tumors. Page 2002, bottom of the left column.

In light of the claim amendments and for the reasons stated above, the claimed invention was not obvious to one of ordinary skill in the art at the time the invention was made, and removal of the rejections under 35 U.S.C. §103(a) is respectfully requested.

SUMMARY

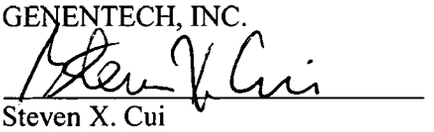
Claims 43-47; 49-59 and new claim 60 are pending in the application.

If in the opinion of the Examiner, a **telephone conference** would expedite the prosecution of the subject application, the Examiner is **strongly encouraged** to call the undersigned at the number indicated below.

This response/amendment is submitted with a transmittal letter and petition for a three month extension of time and fees. In the unlikely event that this document is separated from the transmittal letter or if fees are required, applicants petition the Commissioner to authorize charging our Deposit Account 07-0630 for any fees required or credits due and any extensions of time necessary to maintain the pendency of this application.

Respectfully submitted,

GENENTECH, INC.

Date: July 17, 2003 By: 

Steven X. Cui
Reg. No. 44,637
Telephone No. (650) 225-8674

PATENT TRADEMARK OFFICE

Clean Set of All Pending Claims

(July 17, 2003)

43. (Currently amended) A method for inhibiting VEGF-induced angiogenesis in a subject, comprising administering to said subject an effective amount of a humanized anti-VEGF antibody which (a) binds human VEGF with a K_d value of no more than about 1 x 10^{-8}M; (b) has an ED50 value of no more than about 5nM for inhibiting VEGF-induced proliferation of endothelial cells *in vitro*; and (c) inhibits VEGF-induced angiogenesis *in vivo*, wherein 5mg/kg of said humanized antibody inhibits at least about 50% of tumor growth in an A673 *in vivo* tumor model.

44. (Currently amended) The method of claim 43, wherein said humanized anti-VEGF antibody binds human VEGF with a K_d value of no more than about 1 x 10^{-9}M.

45. (Previously presented) The method of claim 43, wherein said subject has a tumor.

46. (Currently amended) The method of claim 45, wherein 5mg/kg of said humanized antibody inhibits at least about 80% of tumor growth in an A673 *in vivo* tumor model.

47. (Currently amended) The method of claim 43, said humanized anti-VEGF antibody having a heavy chain and a light chain, wherein the heavy chain variable domain comprises four framework regions (FR) and three complementarity determining regions (CDR) as a contiguous sequence represented by the formula: FR1-CDRH1-FR2-CDRH2-FR3-CDRH3-FR4, wherein the four FRs are derived from a consensus human antibody heavy chain framework region sequence and the three CDRs are derived from a non-human anti-VEGF antibody, and wherein the light chain variable domain comprises four FRs and three CDRs as a contiguous sequence represented by the formula: FR1-CDRL1-FR2-CDRL2-FR3-CDRL3-FR4, wherein the four FRs are derived from a consensus human antibody light chain framework region sequence and the three CDRs are derived from the non-human anti-VEGF antibody.

49. (Currently amended) The method of claim 47, wherein the heavy chain variable domain comprises the following CDR amino acid sequences: CDRH1 (GYX_1FTX_2YGMN, wherein X_1 is T or D and X_2 is N or H; SEQ ID NO: 128), CDRH2 (WINTYTGEPTYAADFKR; SEQ ID

NO:2) and CDRH3 ($YPX_1YYGX_2SHWYFDV$, wherein X_1 is Y or H and X_2 is S or T; SEQ ID NO: 129); and wherein the light chain variable domain comprises the following CDR amino acid sequences: CDRL1 (SASQDISNYLN; SEQ ID NO:4), CDRL2 (FTSSLHS SEQ ID NO:5) and CDRL3 (QQYSTVPWT; SEQ ID NO:6).

50. (Currently amended) The method of claim 47, wherein the heavy chain variable domain comprises the following CDR amino acid sequences: CDRH1 (GYTFTNYGMN; SEQ ID NO:1), CDRH2 (WINTYTGEPTYAADFKR; SEQ ID NO:2) and CDRH3 (YPHYYGSSHWYFDV; SEQ ID NO:3); and wherein the light chain variable domain comprises the following CDR amino acid sequences: CDRL1 (SASQDISNYLN; SEQ ID NO:4), CDRL2 (FTSSLHS SEQ ID NO:5) and CDRL3 (QQYSTVPWT; SEQ ID NO:6).

51. (Currently amended) A method for inhibiting VEGF-induced angiogenesis in a subject, comprising administering to said subject an effective amount of a humanized anti-VEGF antibody which binds human VEGF with a K_d value of no more than about 1×10^{-8}M, said humanized anti-VEGF antibody comprising a heavy chain variable domain sequence of SEQ ID NO:116 and a light chain variable domain sequence of SEQ ID NO:115.

52. (Currently amended) A method for inhibiting VEGF-induced angiogenesis in a subject, comprising administering to said subject an effective amount of a humanized anti-VEGF antibody which binds human VEGF with a K_d value of no more than about 1×10^{-8}M, said humanized anti-VEGF antibody comprising a heavy chain variable domain sequence of SEQ ID NO:7 and a light chain variable domain sequence of SEQ ID NO:8.

53. (Previously presented) The method of claim 43, wherein said humanized anti-VEGF antibody is a full length antibody.

54. (Previously presented) The method of claim 53, wherein said humanized anti-VEGF antibody is a human IgG.

55. (Previously presented) The method of claim 43, wherein said humanized anti-VEGF antibody is an antibody fragment.

56. (Previously presented) The method of claim 55, wherein said humanized anti-VEGF antibody is a Fab.

57. (Previously presented) The method of claim 43, wherein said subject has a retinal disease.

58. (Previously presented) The method of claim 57, wherein said retinal disease is age-related macular degeneration (AMD).

59. (Previously presented) The method of claim 58, wherein the humanized anti-VEGF antibody is administered to the subject at a dose of at least about 0.5mg/kg.

60. (New) The method of claim 47, wherein the heavy chain variable domain FR has at least one substitution wherein the human FR residue is replaced by a corresponding residue from the non-human anti-VEGF antibody, said residue is selected from the following positions: 37H, 49H, 67H, 69H, 71H, 73H, 75H, 76H, 78H and 94H; and wherein the light chain variable domain FR has at least one substitution wherein the human FR residue is replaced by a corresponding residue from the non-human anti-VEGF antibody, said residue is selected from the following positions: 4L, 46L and 71L (positions according to Kabat numbering).

0718-03

1642 PrtOfc

Application No. 09/723752

NOTICE TO COMPLY WITH REQUIREMENTS FOR PATENT APPLICATIONS CONTAINING NUCLEOTIDE SEQUENCE AND/OR AMINO ACID SEQUENCE DISCLOSURES

The nucleotide and/or amino acid sequence disclosure contained in this application does not comply with the requirements for such a disclosure as set forth in 37 C.F.R. 1.821 - 1.825 for the following reason(s):

☐ 1. This application clearly fails to comply with the requirements of 37 C.F.R. 1.821-1.825. Applicant's attention is directed to these regulations, published at 1114 OG 29, May 15, 1990 and at 55 FR 18230, May 1, 1990.

☐ 2. This application does not contain, as a separate part of the disclosure on paper copy, a "Sequence Listing" as required by 37 C.F.R. 1.821(c).

☐ 3. A copy of the "Sequence Listing" in computer readable form has not been submitted as required by 37 C.F.R. 1.821(e).

☐ 4. A copy of the "Sequence Listing" in computer readable form has been submitted. However, the content of the computer readable form does not comply with the requirements of 37 C.F.R. 1.822 and/or 1.823, as indicated on the attached copy of the marked -up "Raw Sequence Listing."

☒ 5. The computer readable form that has been filed with this application has been found to be damaged and/or unreadable as indicated on the attached CRF Diskette Problem Report. A Substitute computer readable form must be submitted as required by 37 C.F.R. 1.825(d).

☐ 6. The paper copy of the "Sequence Listing" is not the same as the computer readable from of the "Sequence Listing" as required by 37 C.F.R. 1.821(e).

☐ 7. Other: _____

Applicant Must Provide:

☒ An initial or substitute computer readable form (CRF) copy of the "Sequence Listing".

☐ An initial or substitute paper copy of the "Sequence Listing", as well as an amendment directing its entry into the specification.

☒ A statement that the content of the paper and computer readable copies are the same and, where applicable, include no new matter, as required by 37 C.F.R. 1.821(e) or 1.821(f) or 1.821(g) or 1.825(b) or 1.825(d).

For questions regarding compliance to these requirements, please contact:

For Rules Interpretation, call (703) 308-4216
For CRF Submission Help, call (703) 308-4212
For PatentIn software help, call (703) 308-6856

PLEASE RETURN A COPY OF THIS NOTICE WITH YOUR RESPONSE

Patent Docket P1093P1D1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Manuel Baca et al. Serial No.: 09/723,752 Filed: November 27, 2000 For: ANTI-VEGF ANTIBODIES	Group Art Unit: 1642 Examiner: Helms, Larry Ronald EXPRESS MAIL LABEL NO.: EV 351 926 729 US DATE OF DEPOSIT: JULY 17, 2003

PETITION AND FEE FOR THREE MONTH EXTENSION OF TIME
(37 CFR 1.136(a))

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Applicant petitions the Commissioner of Patents and Trademarks to extend the time for response to the Office Action dated January 17, 2003 for three (3) month(s) from April 17, 2003 to July 17, 2003. The extended time for response does not exceed the statutory period.

Please charge Deposit Account No. 07-0630 in the amount of $930 to cover the cost of the extension. Any deficiency or overpayment should be charged or credited to this deposit account. A duplicate of this sheet is enclosed.

Respectfully submitted,
GENENTECH, INC.

Date: July 17, 2003

By: _____
Steven X. Cui
Reg. No. 44,637
Telephone No. (650) 225-8674

RAW SEQUENCE LISTING DATE: 07/28/2003
PATENT APPLICATION: **US/09/723,752B** TIME: 09:47:07

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Output Set: **N:\CRF4\07282003\I723752B.raw**

SEQUENCE LISTING

W--> 3 **SEQUENCE LISTING**
5 (1) GENERAL INFORMATION:
7 (i) APPLICANT: Baca, Manuel
8 Wells, James A.
9 Presta, Leonard G.
10 Lowman, Henry B.
11 Chen, Yvonne M.
13 (ii) TITLE OF INVENTION: ANTI-VEGF ANTIBODIES
15 (iii) NUMBER OF SEQUENCES: 131
17 (iv) CORRESPONDENCE ADDRESS:
18 (A) ADDRESSEE: Genentech, Inc.
19 (B) STREET: 1 DNA Way
20 (C) CITY: South San Francisco
21 (D) STATE: California
22 (E) COUNTRY: USA
23 (F) ZIP: 94080
25 (v) COMPUTER READABLE FORM:
26 (A) MEDIUM TYPE: 3.5 inch, 1.44 Mb floppy disk
27 (B) COMPUTER: IBM PC compatible
28 (C) OPERATING SYSTEM: PC-DOS/MS-DOS
29 (D) SOFTWARE: WinPatin (Genentech)
31 (vi) CURRENT APPLICATION DATA:
C--> 32 **(A) APPLICATION NUMBER: US/09/723,752B**
C--> 33 **(B) FILING DATE: 27-Nov-2000**
34 (C) CLASSIFICATION:
40 (vii) PRIOR APPLICATION DATA:
37 (A) APPLICATION NUMBER: 08/908469
38 (B) FILING DATE: 06-AUG-1997
41 (A) APPLICATION NUMBER: 08/833504
42 (B) FILING DATE: 07-APR-1997
44 (viii) ATTORNEY/AGENT INFORMATION:
45 (A) NAME: Cui, Steven X.
46 (B) REGISTRATION NUMBER: 44,637
47 (C) REFERENCE/DOCKET NUMBER: P1093P1D1
49 (ix) TELECOMMUNICATION INFORMATION:
50 (A) TELEPHONE: 650/225-8674
51 (B) TELEFAX: 650/952-9881
52 (2) INFORMATION FOR SEQ ID NO: 1:
54 (i) SEQUENCE CHARACTERISTICS:
55 (A) LENGTH: 10 amino acids
56 (B) TYPE: Amino Acid
57 (D) TOPOLOGY: Linear

RAW SEQUENCE LISTING DATE: 07/28/2003
PATENT APPLICATION: **US/09/723,752B** TIME: 09:47:07

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67            (A) LENGTH: 17 amino acids
68            (B) TYPE: Amino Acid
69            (D) TOPOLOGY: Linear
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76  Lys Arg
79  (2) INFORMATION FOR SEQ ID NO: 3:
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120           (D) TOPOLOGY: Linear
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RAW SEQUENCE LISTING DATE: 07/28/2003
PATENT APPLICATION: **US/09/723,752B** TIME: 09:47:07

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35 40 45
Glu Trp Val Gly Trp Ile Asn Thr Tyr Thr Gly Glu Pro Thr Tyr
50 55 60
Ala Ala Asp Phe Lys Arg Arg Phe Thr Phe Ser Leu Asp Thr Ser
65 70 75
Lys Ser Thr Ala Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
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Thr Ala Val Tyr Tyr Cys Ala Lys Tyr Pro His Tyr Tyr Gly Ser
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(A) LENGTH: 110 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

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35 40 45
Val Leu Ile Tyr Phe Thr Ser Ser Leu His Ser Gly Val Pro Ser
50 55 60
Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
65 70 75
Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
80 85 90
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Ile Lys Arg Thr Val
110

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(A) LENGTH: 123 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

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20 25 30

RAW SEQUENCE LISTING DATE: 07/28/2003
PATENT APPLICATION: **US/09/723,752B** TIME: 09:47:07

Input Set : **A:\P1093P1D1.txt**
Output Set: **N:\CRF4\07282003\I723752B.raw**

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214  Ala Ala Asp Phe Lys Arg Arg Phe Thr Phe Ser Leu Glu Thr Ser
215                  65                  70                  75
217  Ala Ser Thr Ala Tyr Leu Gln Ile Ser Asn Leu Lys Asn Asp Asp
218                  80                  85                  90
220  Thr Ala Thr Tyr Phe Cys Ala Lys Tyr Pro His Tyr Tyr Gly Ser
221                  95                 100                 105
223  Ser His Trp Tyr Phe Asp Val Trp Gly Ala Gly Thr Thr Val Thr
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226  Val Ser Ser
229 (2) INFORMATION FOR SEQ ID NO: 10:
231      (i) SEQUENCE CHARACTERISTICS:
232           (A) LENGTH: 108 amino acids
233           (B) TYPE: Amino Acid
234           (D) TOPOLOGY: Linear
236     (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:
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241  Gly Asp Arg Val Ile Ile Ser Cys Ser Ala Ser Gln Asp Ile Ser
242                  20                  25                  30
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247  Val Leu Ile Tyr Phe Thr Ser Ser Leu His Ser Gly Val Pro Ser
248                  50                  55                  60
250  Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Tyr Ser Leu Thr Ile
251                  65                  70                  75
253  Ser Asn Leu Glu Pro Glu Asp Ile Ala Thr Tyr Tyr Cys Gln Gln
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266           (B) TYPE: Amino Acid
267           (D) TOPOLOGY: Linear
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283  Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser
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RAW SEQUENCE LISTING DATE: 07/28/2003
PATENT APPLICATION: US/09/723,752B TIME: 09:47:07

Input Set : A:\P1093P1D1.txt
Output Set: N:\CRF4\07282003\I723752B.raw

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          (B) TYPE: Amino Acid
          (D) TOPOLOGY: Linear
    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:
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                   20                  25                  30
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                   35                  40                  45
 Leu Leu Ile Tyr Ala Ala Ser Ser Leu Glu Ser Gly Val Pro Ser
                   50                  55                  60
 Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
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 Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
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          (B) TYPE: Amino Acid
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                   20                  25                  30
 Asn Tyr Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
                   35                  40                  45
 Leu Leu Ile Tyr Phe Thr Ser Ser Leu His Ser Gly Val Pro Ser
                   50                  55                  60
 Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
                   65                  70                  75
 Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
                   80                  85                  90
 Tyr Ser Thr Val Pro Trp Thr Phe Gly Gln Gly Thr Lys Val Glu
                   95                 100                 105
 Ile Lys
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/723,752	11/27/2000	Manuel Baca	P1093P1D1	6340

9157 7590 09/26/2003

GENENTECH, INC.
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EXAMINER
HELMS, LARRY RONALD

ART UNIT	PAPER NUMBER
1642	18

DATE MAILED: 09/26/2003

Please find below and/or attached an Office communication concerning this application or proceeding.

PTO-90C (Rev. 07-01)

Office Action Summary	Application No.	Applicant(s)	
	09/723,752	BACA ET AL.	
	Examiner	Art Unit	
	Larry R. Helms	1642	

-- The MAILING DATE of this communication appears on the cover sheet with the c rrespondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133).
- Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *17 July 2003* .

2a)☒ This action is **FINAL**. 2b)☐ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *43-47 and 49-60* is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☒ Claim(s) *51 and 52* is/are allowed.

6)☒ Claim(s) *47,49,50 and 53-60* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

11)☐ The proposed drawing correction filed on _____ is: a)☐ approved b)☐ disapproved by the Examiner.

If approved, corrected drawings are required in reply to this Office action.

12)☐ The oath or declaration is objected to by the Examiner.

Priority under 35 U.S.C. §§ 119 and 120

13)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____ .

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

14)☐ Acknowledgment is made of a claim for domestic priority under 35 U.S.C. § 119(e) (to a provisional application).

a) ☐ The translation of the foreign language provisional application has been received.

15)☒ Acknowledgment is made of a claim for domestic priority under 35 U.S.C. §§ 120 and/or 121.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO-1449) Paper No(s) _____ .
4) ☐ Interview Summary (PTO-413) Paper No(s). _____ .
5) ☐ Notice of Informal Patent Application (PTO-152)
6) ☐ Other:

DETAILED ACTION

1. Claims 43-44, 46-47, 49-52 have been amended.

Claim 60 has been added.

Claims 43-47, 49-60 are pending and under examination.

2. The text of those sections of Title 35 U.S.C. code not included in this office action can be found in a prior Office Action.

3. The following Office Action contains NEW GROUNDS of rejection.

Rejections Withdrawn

4. The rejection of claims 47, 49-50 under 35 U.S.C. 112, first paragraph, is withdrawn in view of the amendments to the claims.

5. The rejection of claims 47, 49-50, 53-56 under 35 U.S.C. 103(a) as being unpatentable over Ferrara et al (WO 94/10202, published 5/11/94) and further in view of Adair et al (WO 91/09967, published 7/11/91) and Yelton et al (The Journal of Immunology 155:1994-2004, 1995) and as evidenced from the specification is withdrawn in view of the new grounds of rejection and amendments to the claims.

6. The rejection of claims 47, 49-50, under 35 U.S.C. 103(a) as being unpatentable over Ferrara et al (WO 94/10202, published 5/11/94) and further in view of Adair et al (WO 91/09967, published 7/11/91) and Yelton et al (The Journal of Immunology 155:1994-2004, 1995) and as evidenced from the specification as applied to claims 43-47, 49-50, 53-56 above, and further in view of Lopez et al (Invest Opthal. And Visual

Science 37:855, 4/96) is withdrawn in view of the amendments tot he claims and the new grounds of rejection.

Response to Arguments

Priority

7. The instant application claims priority to provisional application 60/126,446, filed 4/7/97. Claims 43, recites the limitation of "a Kd value of no more than about 1 X 10-8M" and claims 43 and46 recites the limitation of "in an A673 in vivo tumor model". The limitations have support in the instant application, however, it appears that there is not support for these limitations in the 60/126,446 application. As such the priority date granted to claims 43-47, 49-60 is 8/6/97.

8. The rejection of Claims 43-46, 53-56 under 35 U.S.C. 103(a) as being unpatentable over Ferrara et al (WO 94/10202, published 5/11/94) and further in view of Adair et al (WO 91/09967, published 7/11/91) and Yelton et al (The Journal of Immunology 155:1994-2004, 1995) and as evidenced from the specification is maintained.

The response filed 7/17/03 has been carefully considured but is deemed not to be persuasive. The response states that by simply applying humanization methods of

Adair et al or Yelton et al to the murine antibodies of Ferrara et al one of ordinary skill in the art would not have had a reasonable expectation of success to produce a humanized anti-VEGF antibody variant that had the binding affinity and the inhibition potencies as currently claimed (see page 9 of response). The response then argues that Adair et al showed that even those with increased binding affinity behaved differently in an unpredictable manner in biological activities. The response then addresses the Yelton et al reference by stating that the binding affinity to LeY does not always correlate with binding affinity to tumor cells with LeY bound on their surface (see page 10-11 of response).

In response to these arguments, while Adair et al does teach a general method, the method can be performed on any antibody and all that is required is the screening of many altered antibodies which would not be undue and would have been routine at the time the claimed invention was made. In addition, while the response is directed to an example in Adair et al where binding to cells were reduce even thought the affinity was increased, there is examples in Adair et al, for example the OKT3 humanized antibody which has good binding to cells and had very similar binding in a competition assay (see pages 36 and 51). Thus, there are examples in Adair et al where high affinity and good in vivo binding is taught. In addition, in the example described in the response which showed a lowering in the ability of the antibody to compete with TNF, it would be obvious that a higher concentration would compete better and this is important in view that the claims require a concentration of 5mg/kg which is at a high dose. In response to the Yelton et al argument, there were several mutant antibodies made and while the

M4 was not optimal, the M3 antibody had improved affinity and better binding to H3396 cells (see page 1999 left column last paragraph). With regard to increasing the affinity may not bring a therapeutic advantage, Yelton et al teach that genetic engineering B72.3 resulted in increased affinity and improved radioactivity delivered to the tumor (see page 2002). Thus, it is obvious that better therapeutic advantage can be expected by optimizing the binding of the antibody as taught by Yelton et al.

Thus, both Adair et al and Yelton et al teach that combining mutations can lead to the desired characteristics. This is important because Ferrera et al teach that the antibodies encompassed in Ferrera et al are those that are humanized and have characteristics of good binding affinity and inhibiting angiogenic activity of VEGF at least about 50%-80% and teach the A673 tumor model (see page 8).

9. The rejection of claims 43-46, 53-59 under 35 U.S.C. 103(a) as being unpatentable over Ferrara et al (WO 94/10202, published 5/11/94) and further in view of Adair et al (WO 91/09967, published 7/11/91) and Yelton et al (The Journal of Immunology 155:1994-2004, 1995) and as evidenced from the specification as applied to claims 43-47, 49-50, 53-56 above, and further in view of Lopez et al (Invest Opthal. And Visual Science 37:855, 4/96) is maintained .

The response filed 7/17/03 has been carefully considured but is deemed not to be persuasive. The response did not address this rejection per se. The response addressed the Adair and Yelton reference above and it is assumed the same arguments

would be applied for this rejection and as such the same response above applies. thus the rejection is maintained.

The following are NEW GROUNDS of rejection

Claim Rejections - 35 USC § 112

10. Claims 47, 49-50 and 60 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

Claims 47 and 49-50 and 60 are indefinite for reciting "FRs are derived" in claim 47. The claims are indefinite for reciting "derived" as the exact meaning of the word is not known. The term "derived" is not one which has a universally accepted meaning in the art nor is it one which has been adequately defined in the specification. The primary deficiency in the use of this phrase is the absence of a ascertainable meaning for said phrase. Since it is unclear how the frameworks are to be derivatized to yield the class of derivatives referred to in the claims, there is no way for a person of skill in the art to ascribe a discrete and identifiable class of compounds to said phrase. Further, it is not clear whether the "derived" framework of the humanized antibody is formed by attachment of a detectable marker, therapeutic molecule, some other molecule or altering the amino acid sequence, for examples. In addition, since the term "derived" does not appear to be clearly defined in the specification, and the term can encompass proteins with amino acid substitutions, insertions, or deletions, antibody fragments,

chemically derivatized molecules, or even antibody mimetics. In absence of a single defined art recognized meaning for the phrase and lacking a definition of the term in the specification, one of skill in the art could not determine the metes and bounds of the claims.

Claim Rejections - 35 USC § 103

11. Claims 43-47, 49-50, 53-60 are rejected under 35 U.S.C. 103(a) as being unpatentable over Ferrara et al (WO 94/10202, published 5/11/94) and further in view of Adair et al (WO 91/09967, published 7/11/91) and Yelton et al (The Journal of Immunology 155:1994-2004, 1995) and as evidenced from the specification and Lopez et al (Invest Opthal. And Visual Science 37:855, 4/96) and Bendig et al (U.S. Patent 5,558,864, 11/92).

The claims recite a method of inhibiting VEGF-induced angiogenesis in a subject by administration of an antibody wherein the subject has a tumor wherein the antibody binds no more than 10-9M, and 5mg/kg inhibits at least 50%-80% of tumor growth in a A673 in vivo model, has an ED50 of no more than 50 nM, the antibody comprises CDRs recited in the claims, the antibody is a full length antibody, a IgG, and a Fab and the FR are derived from a consensus sequence and has at least one substitution at position 49, 71, 73, 75, 76 of the heavy chain and at least one substitution at position 46 or 71 in the light chain and wherein the subject has age related macular degeneration and the antibody is administered at a dose of at least about 0.5 mg/kg.

Ferrara et al teach an anti-VEGF antibody (see abstract). Ferrara et al also teach a humanized antibody (see page 8, lines 13-31) and the effect of the antibodies in tumor cell growth and angiogenesis (see page 23-24 and page 4). Ferrara et al also teach methods of inhibiting VEGF-induced angiogenesis in a subject and the subject can have cancer and the antibody was tested in a A673 model (see abstract and Example 2) and the humanized antibody binds with 10-9M affinity (see page 8). Ferrara et al also teach administration at 0.1 to 100 mg/kg (see page 15). Ferrara et al does not teach a specific method for humanization or obtaining the CDR sequence of the antibody or consensus Fr or AMD. These deficiencies are made up for in the teachings of Adair et al, Yelton et al, Lopez et al, and Bendig et al.

Adair et al teach a method of antibody humainzation by CDR grafting and framework modifications and methods of obtaining the amino acid sequences of antibodies from hybridomas and fragments of the antibody such as Fabs (see abstract and entire document) and substitutions at positions in the heavy and light chains, specifically H49, H71, H75, H76, H78 and L46 and L71 (see abstract).

Yelton et al teach an affinity maturation method comprising alterations in the CDRs of the heavy chain (see abstract).

Lopez et al teach VEGF may be important in the progression of ARMD (see page 865) and VEGF is a critical factor in CNVM development (see page 856).

Bendig et al teach humanization of antibodies using a consensus sequence in the FR as well as substitutions at FR positions.

It would have been prima facie obvious to one of ordinary skill in the art at the time the claimed invention was made to have produced a method of inhibiting VEGF-induced angiogenesis in a subject with AMD by administration of a humanized antibody of Ferrara humanized by the methods of Adair et al, Yelton et al, and Bendig et al in view of Lopez et al.

One of ordinary skill in the art would have been motivated to and had a reasonable expectation of success to produce the claimed method with a humanized anti-VEGF antibody because Adair et al teach "most Mabs are of rodent origin, they are naturally antigenic in humans and thus can give rise to an undesirable immune response termed the HAMA" (see page 2). In addition, one of ordinary skill in the art would have been motivated and had a reasonable expectation of success to produce the claimed method because Ferrara et al teach the antibody can be humanized and the tumors from A4.6.1 treated animals were smaller than those tumors in mice treated with a control antibody (see Figure 5). In addition, one of ordinary skill in the art would have been motivated to and had a reasonable expectation of success to produce the claimed method because Yelton et al teach a method for affinity maturation of an antibody in order to "change the form, affinity, and potentially the specificity of Abs to optimize them for delivering a wide variety of therapeutic agents to tumor cells." (See page 2002 last paragraph). In addition it would have been obvious to humanize the antibody by picking a consensus Fr for the heavy and light chains because Bendig et al teach that effective and specific humanized monoclonal antibodies can be easily obtained by using a consensus sequence)see column 4, lines 13-15) and a consensus

sequence can be synthesized as a whole without problems and there is no dependence on the knowledge or availability of certain individual antibodies (see column 4, lines 38-50).

One of ordinary skill in the art would have been motivated to and had a reasonable expectation of success to produce the claimed method because Ferrera et al teach the methods for inhibition of angiogenesis in a subject with many diseases and in view of Lopez which teaches VEGF is involved in angiogenesis in ARMD it would be obvious to inhibit ARMD with a humanized antibody to VEGF.

Moreover, it would have been obvious to humanize the A4.6.1 antibody of Ferrara et al by the methods of Adair et al, Yelton et al, and Bendig et al because Ferrara et al teach human VEGF and in view of Adair and Yelton et al and Bendig et al it would be obvious to humanize the antibody for therapy for inhibiting VEGf-induced angiogenesis.

As evidenced from the specification the A4.6.1 antibody of Ferrara et al has the CDRs as recited in claims 47, 49-50 (see Figure 1A and 1B of the specification).

It is the Examiner's position that the antibody produced by humanizing Ferrara et al's antibody with Adair et al's and Yelton et al's method would produce a humanized antibody that would have the binding and inhibition characteristics claimed in the claimed method. One of ordinary skill in the art would reasonably conclude that Ferrara et al's antibody humanized with Adair et al's and Yelton et al's method also possesses (1) the same binding affinity to the human VEGF, and (2) inhibits angiogenesis and tumor growth of at least about 50% in A673 in vivo tumor model and has an Ed50 of

5nM, therefore, it appears that Ferrara et al's antibody humanized with Adair et al's, Yelton et al's, and Bendig et al's method would produce a humanized antibody that is identical to the claimed antibody. Since the Patent and Trademark Office does not have the facilities for examining and comparing the claimed humanized antibody with the humanized antibody of Ferrara et al's antibody humanized with Adair et al's, Yelton et al's, and Bendig et al's method, the burden of proof is upon the Applicants to show an unobvious distinction between the structural and functional characteristics of the claimed antibody and the antibody of the prior art. See In re Best, 562 F.2d 1252, 195 U.S.P.Q. 430 (CCPA 197) and Ex parte Gray, 10 USPQ 2d 1922 1923 (PTO Bd. Pat. App. & Int.).

Therefore, the invention as a whole was prima facie obvious to one of ordinary skill in the art at the time the invention was made, as evidenced by the references.

Conclusion

12. Claims 51 and 52 are in condition for allowance.

13. Applicant's amendment necessitated the new ground(s) of rejection presented in this Office action. Accordingly, **THIS ACTION IS MADE FINAL**. See MPEP § 706.07(a). Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the date of this final action.

14. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Larry R. Helms, Ph.D, whose telephone number is (703) 306-5879. The examiner can normally be reached on Monday through Friday from 7:00 am to 4:30 pm, with alternate Fridays off. If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Anthony Caputa, can be reached on (703) 308-3995. Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to the Group receptionist whose telephone number is (703) 308-0196.

15. Papers related to this application may be submitted to Group 1600 by facsimile transmission. Papers should be faxed to Group 1600 via the PTO Fax Center located in Crystal Mall 1. The faxing of such papers must conform with the notice published in the Official Gazette, 1096 OG 30 (November 15, 1989). The CM1 Fax Center telephone number is (703) 308-4242.

Respectfully,

Larry R. Helms Ph.D.

703-306-5879

LARRY R. HELMS, PH.D
PRIMARY EXAMINER

Notice of References Cited	Application/Control No. 09/723,752	Applicant(s)/Patent Under Reexamination BACA ET AL.	
	Examiner Larry R. Helms	Art Unit 1642	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
	A	US-5,558,864	09-1996	Bendig et al	-----
	B	US-			
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	
	V	
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

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AF/1642

Patent Docket P1093P1D1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Manuel Baca et al. Serial No.: 09/723,752 Filed: November 27, 2000 For: ANTI-VEGF ANTIBODIES	Group Art Unit: 1642 Examiner: Helms, Larry Ronald Confirmation No: 6340 CUSTOMER NO: 09157

CERTIFICATE OF MAILING

I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450 on

October 30, 2003

T. G. Marrs

Tom Marrs

SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Applicants submit herewith patents, publications or other information (attached hereto and listed on the attached revised Form PTO-1449) of which they are aware, which they believe may be material to the examination of this application and in respect of which there may be a duty to disclose in accordance with 37 CFR §1.56.

This Information Disclosure Statement is filed in accordance with the provisions of:

[] **37 CFR §1.97(b)**

- within three months of the filing date of the application other than a continued prosecution application under 37 CFR §1.53(d); **or**
- within three months of the date of entry of the national stage of a PCT application as set forth in 37 CFR§1.491, **or**
- before the mailing of the first Office action on the merits; **or**
- before the mailing of the first Office action after the filing of a request for a continued examination under 37 CFR §1.114.

[] **37 CFR §1.97(c)**

- by the applicant after the period specified in 37 CFR §1.97(b), but prior to the

mailing date of any of a final action under 37 CFR §1.113, or a notice of allowance under 37 CFR §1.311, or an action that otherwise closes prosecution in the application, and is accompanied by either the fee set forth in 37 CFR §1.17(p) **or** a statement as specified in 37 CFR §1.97(e), as checked below.

[X] **37 CFR §1.97(d)**

- after the period specified in CFR §1.97(c), and is accompanied by the fee set forth in 37 CFR §1.17(p) **and** a statement as specified in 37 CFR §1.97(e), as checked below.

[If either of boxes 37 CFR §1.97(c) or 37 CFR §1.97(d) is checked above, the following statement under 37 CFR §1.97(e) may need to be completed.]

[X] **37 CFR §1.97(e)**Each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of this information disclosure statement.

[X] **37 CFR §1.704(d)** Each item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application and the communication was not received by any individual designated in §1.56(c) more than thirty days prior to the filing of this information disclosure statement. Therefore, in accordance with the provisions of 37 CFR §1.704(d), the filing of this information disclosure statement will not be considered a failure to engage in reasonable efforts to conclude prosecution under 37 CFR §1.704.

[X] The U.S. Patent and Trademark Office is hereby authorized to charge Deposit Account No. 07-0630 in the amount of $180.00 to cover the cost of this Information Disclosure Statement under 37 CFR §1.17(p). Any deficiency or overpayment should be charged or credited to this deposit account.

A list of the patent(s) or publication(s) is set forth on the attached revised Form PTO-1449 (Modified). A copy of the items on PTO-1449 is supplied herewith.

Those patent(s) or publication(s) which are marked with an asterisk (*) in the attached PTO-1449 form are not supplied because they were previously cited by or submitted to the Office in a prior application Serial No. 08/908,469, filed August 6, 1997 and relied upon in this application for an earlier filing date under 35 USC §120.

A concise explanation of relevance of the items listed on PTO-1449 is:

[X] not given

[] given for each listed item

[] given for only non-English language listed item(s) [Required]

[] in the form of an English language copy of a Search Report from a foreign patent office, issued in a counterpart application, which refers to the relevant portions of the references.

In accordance with 37 CFR §1.97(g), the filing of this information disclosure statement shall not be construed as a representation that a search has been made.

In accordance with 37 CFR §1.97(h), the filing of this information disclosure statement shall not be construed to be an admission that the information cited in the statement is, or is considered to be, material to patentability as defined in 37 CFR § 1.56(b).

The Commissioner is hereby authorized to charge any additional fees required under 37 CFR 1.16 and 1.17 for this Information Disclosure Statement, or credit overpayment to Deposit Account No. 07-0630. A duplicate copy of this sheet is enclosed.

Respectfully submitted,

GENENTECH, INC.

Date: October 20, 2003

By: _____

Steven X. Cui
Reg. No. 44,637
Telephone No. (650)225- 8674

FORM PTO-1449

U.S. Dept. of Commerce
Patent and Trademark Office

Atty Docket No.	Serial No.
P1093P1D1	09/723752
Applicant: Baca et al.	
Filing Date: 27 Nov 2000	Group: 1642

LIST OF DISCLOSURES CITED BY APPLICANT

(Use several sheets if necessary)

U.S. PATENT DOCUMENTS

Examiner Initials		Document Number	Date	Name	Class	Subclass	Filing Date
	79	2002/032315	14.03.02	Baca et al.			
	80	6,037,454	14.03.00	Jardieu et al.			

FOREIGN PATENT DOCUMENTS

Examiner Initials		Document Number	Date	Country	Class	Subclass	Translation Yes	Translation No
	81	WO 98/45331	15.10.98	PCT				
	82	WO 98/45332	15.10.98	PCT				

OTHER DISCLOSURES (Including Author, Title, Date, Pertinent Pages, etc.)

	83	Presta et al., "Humanization of an Anti-Vascular Endothelial Growth Factor Monoclonal Antibody for the Therapy of Solid Tumors and Other Disorders" Cancer Research 57(20):4593-4599 (Oct 15, 1997)

Examiner	Date Considered

*Examiner: Initial if reference considered, whether or not citation is in conformance with MPEP 609; draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

USCOMM-DC 80-398.

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(11) International Publication Number: **WO 98/45332**

(43) International Publication Date: 15 October 1998 (15.10.98)

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(30) Priority Data:
08/833,504 7 April 1997 (07.04.97) US

(71) Applicant *(for all designated States except US)*: GENENTECH, INC. [US/US]; One DNA Way, South San Francisco, CA 94080 (US).

(72) Inventors; and
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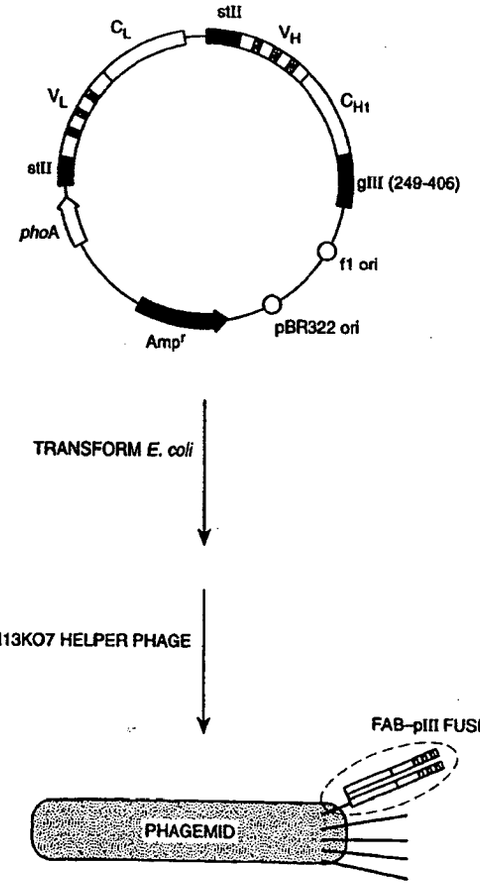
(54) Title: HUMANIZED ANTIBODIES AND METHODS FOR FORMING HUMANIZED ANTIBODIES

(57) Abstract

Described herein is a humanized antibody to vascular endothelial growth factor (VEGF). Also described herein is a method for rapidly producing and identifying framework mutations which improve the binding of humanized antibodies to their cognate antigens. In a preferred embodiment, non-human CDRs are grafted onto a human $V_I\kappa I$–$V_H III$ framework. Random mutagenesis of a small set of critical framework residues is also performed followed by monovalent display of the resultant library of antibody molecules on the surface of filamentous phage. The optimal framework sequences are then identified by affinity-based selection. Optionally, the selected antibodies can be further mutated so as to replace vernier residues which sit at the V_L–V_H interface by residues which match the non-human parent antibody. The methods described herein can be applied to any non-human antibody. Accordingly, humanized antibodies are provided.



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HUMANIZED ANTIBODIES AND METHODS FOR FORMING HUMANIZED ANTIBODIES

FIELD OF THE INVENTION

The present invention is directed at humanized antibodies and methods for preparing humanized antibodies. In particular, the present invention is directed at methods for preparing humanized antibodies using a monovalent phage display system and antibody mutants produced by random mutagenesis of a small set of critical framework residues made to a single human framework. More particularly, this invention is directed at the humanization of a murine antibody which binds to vascular endothelial growth factor (VEGF).

BACKGROUND OF THE INVENTION

Monoclonal antibodies (mAbs) have enormous potential as therapeutic agents, particularly when they can be used to regulate defined systems. For example, in some circumstances it would be desirable to regulate a system such as angiogenesis, where new blood capillaries are formed from the walls of existing small vessels. Angiogenesis is generally important after infliction of a wound or infection so that a burst of capillary growth can be stimulated in the neighborhood of the damaged tissue. However, angiogenesis is also important in tumor growth since, for continued growth, a tumor must induce the formation of a capillary network that invades the tumor mass.

Certain growth factors have been identified which regulate angiogenesis. Of particular interest is the vascular endothelial growth factor (VEGF), which seems to be the agent by which some tumors acquire their rich blood supply. Molecular Biology of the Cell, 3rd Ed., Alberts et al., Garland Publishing, page 1154 (1994). Therefore, mAbs to VEGF, for example, can be useful for a variety of reasons, including for use in the regulation of angiogenesis and more particularly, as an anti-tumor agent. A murine anti-VEGF mAb A4.6.1 which blocks VEGF receptor binding has been previously described. This antibody has been shown to inhibit mitogenic signaling. Kim et al., *Growth Factors* 7, 53 (1992); Kim et al., *Nature* 362, 841 (1993).

Most mAbs including the anti-VEGF described above are derived from murine or other non-human sources which limits clinical efficacy. In particular, the body often reacts with an immunogenic response to non-human antibodies whereby the antibody is rapidly cleared from the system before any therapeutic effect can occur. In addition to the immunogenicity of non-human mAbs invoked when administered to humans, further limitations arise from weak recruitment of effector function.

As a means of circumventing these deficiencies, the antigen binding properties of non-human mAbs can be conferred to human antibodies through a process known as antibody "humanization". A humanized antibody contains the amino acid sequence from the six complementarily-determining regions (CDRs) (the antigen-binding site of the antibody molecule) of the parent or corresponding non-human mAb, grafted onto a human antibody framework. Therefore, humanization of non-human antibodies is commonly referred to as CDR grafting. The low content of non-human sequence in such humanized antibodies (~5%) has proven effective in reducing the immunogenicity and prolonging the serum half-life of the antibodies administered to humans. Inter alia, humanized monoclonal antibodies ("chimeric immunoglobulins") are disclosed in U.S. Patent No. 4,816,567.

Unfortunately, simple grafting of CDR sequences often yields humanized antibodies which bind antigen much more weakly than the parent non-human mAb. In order to restore high affinity, the antibody must be further engineered to fine-tune the structure of the antigen binding loops. This is achieved by replacing key residues in the framework regions of the antibody variable domains with the matching sequence from the parent murine antibody. These framework residues are usually involved in supporting the conformation of the CDR loops, although some framework residues may themselves directly contact the antigen. Studies have been conducted which note the importance of certain framework residues to CDR conformation and a comprehensive list of all the framework residues which can affect antigen binding has been compiled. Chothia et al., *J. Mol. Biol.* 224, 487 (1992); Foote et al., *J. Mol. Biol.* 224, 489 (1992). The comprehensive list includes some thiry "vernier" residues which can potentially contribute to CDR structure. Although higher antigen affinity would likely result from editing the entire set of vernier residues within a humanized antibody so as to match the corresponding parent non-human sequence, this is not generally desirable

given the increased risk of immunogenicity imposed by adding further elements of non-human sequence. Thus, from a therapeutic standpoint, it is preferable to confine framework changes to the minimum set which affords a high affinity humanized antibody.

Therefore, it is desirable to identify a small set of changes which suffice to optimize binding, however, the required changes are expected to differ from one humanized antibody to the next. To achieve the desired result, one approach has been to identify the proper combination of mutations by constructing a panel of mutants having "suspect" framework residues replaced by their murine counterpart. These variants are each individually formed and tested for antigen and then combined with other variants found to have favorable binding affinities. However, this method involves cycles of individual site-directed mutagenesis, isolation and screening, and is therefore undesirable because it is time consuming and tedious.

As a means of simplifying antibody humanization, a number of different approaches have been developed. See, for example, Queen et al., *PNAS USA* 86, 10029 (1989); Kettleborough et al., *Protein Eng.* 4, 773 (1991); Tempest et al., *Biotechnology* 9, 266 (1991); Padlan, *Mol. Immunol.* 28, 489 (1991); Roguska et al., *PNAS USA* 91, 969 (1994); Studnicka et al., *Protein Eng.* 7, 805 (1994); Allen et al., *J. Immunol.* 135, 368 (1985); Carter et al., *PNAS USA* 89, 4285 (1992); Presta et al., *J. Immunol.* 151, 2623 (1993); Eigenbrot et al., *Proteins* 18, 49 (1994); Shalaby et al., *J. Exp. Med.* 175, 217 (1992); Kabat et al., Sequences of Proteins of Immunological Interest, (5th), Public Health Service, NIH, Bethesda, MD (1991); and Rosok et al., *J. Biol. Chem.* 271, 22611 (1996).

It is an object of the present invention to provide a general means of rapidly selecting framework mutations which improve the binding of humanized antibodies to their cognate antigens wherein the current methods of framework optimization based on cycles of individual site-directed mutagenesis and screening are eliminated.

It is also an object to provide rapid methods of humanizing antibodies which provide antibodies with low immunogenecity and which utilize a single human framework as a generic scaffold.

It is a further object of the present invention to provide humanized antibodies which are mutated to have enhanced affinity for antigen relative to the initial humanized antibody with no framework changes.

It is additionally a further object of the present invention to provide humanized antibodies that have a reduced clearance rate and hence longer retention within the body after systemic administration such that lower doses of the material are available for systemic administration for therapeutic effect.

It is also a further object of the present invention to provide humanized monoclonal antibodies to VEGF.

SUMMARY OF THE INVENTION

The present invention provides a humanized antibody to vascular endothelial growth factor (VEGF). The initial humanized anti-VEGF has a framework derived from consensus sequences of the most abundant human subclasses, namely $V_L\kappa$ subgroup I ($V_I\kappa I$) and V_H subgroup III (V_HIII) wherein the CDRs from non-human anti-VEGF are grafted thereon. Random mutagenesis of critical framework residues on the initial construct produced the humanized anti-VEGF described herein which has 125 fold enhanced affinity for antigen relative to the initial humanized antibody with no framework changes. A single additional mutation gave a further six fold improvement in binding. This humanized anti-VEGF can be reproduced by the method described herein or by traditional recombinant techniques given the sequence information provided herein.

Also provided herein is a method for rapidly producing and identifying framework mutations which improve the binding of humanized antibodies to their cognate antigens. In a preferred embodiment, non-human CDRs are grafted onto a human $V_I\kappa I$- V_HIII framework. Random mutagenesis of a small set of critical framework residues is also performed followed by monovalent display of the resultant library of antibody molecules on the surface of filamentous phage. The optimal framework sequences are then identified by affinity-based selection. Optionally, the selected antibodies can be further mutated so as to replace vernier

residues which sit at the V_L-V_H interface with residues which match the non-human parent antibody.

The methods described herein can be applied to any non-human antibody. Accordingly, humanized antibodies are provided by the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the amino acid sequences of murine A4.6.1 (SEQ ID NO: 6 and 9 for the V_L and V_H domains, respectively), humanized A4.6.1 variant hu2.0, (SEQ ID NO: 7 and 10 for the V_L and V_H domains, respectively), and humanized A4.6.1 variant hu2.10 (SEQ ID NO: 8 and 11 for the V_L and V_H domains, respectively). Sequence numbering is according to Kabat et al., Sequences of Proteins of Immunological Interest, (5th), Public Health Service, NIH, Bethesda, MD (1991) and mismatches are indicated by asterisks (murine A4.6.1 vs hu2.0) or bullets (hu2.0 vs hu2.10). Variant hu2.0 contains only the CDR sequences (bold) from the murine antibody grafted onto a human light chain K subgroup I, heavy chain subgroup III framework. Variant hu2.10 is the consensus humanized clone obtained from phage sorting experiments described herein.

Figure 2 depicts the framework residues targeted for randomization.

Figure 3 depicts the phagemid construct for surface display of Fab-pIII fusions on phage. The phagemid construct encodes a humanized version of the Fab fragment for antibody A4.6.1 fused to a portion of the M13 gene III coat protein. The fusion protein consists of the Fab joined at the carboxyl terminus of the heavy chain to a single glutamine residue (from suppression of an amber codon in supE *E. coli*), then the C-terminal region of the gene III protein (residues 249-406). Transformation into F^+ *E. coli*, followed by superinfection with M13KO7 helper phage, produces phagemid particles in which a small proportion of these display a single copy of the fusion protein.

Detailed Description of the Invention:

A. Definitions

"Antibodies" (Abs) and "immunoglobulins" (Igs) are glycoproteins having the same structural

characteristics. While antibodies exhibit binding specificity to a specific antigen, immunoglobulins include both antibodies and other antibody-like molecules which lack antigen specificity. Polypeptides of the latter kind are, for example, produced at low levels by the lymph system and at increased levels by myelomas.

"Native antibodies" and "native immunoglobulins" are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies between the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. Each light chain has a variable domain at one and (V_L) and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light and heavy chain variable domains. Clothia et al., *J. Mol. Biol.* 186, 651 (1985); Novotny et al., *PNAS USA* 82, 4592 (1985).

The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed through the variable domains of antibodies. It is concentrated in three segments called "complementarily determining regions" (CDRs) or "hypervariable regions" both in the light chain and the heavy chain variable domains. The more highly conserved portions of variable domains are called the framework (FR). The variable domains of native heavy and light chains each comprise four FR regions, largely adopting a p-sheet configuration, connected by three CDRs, which form loops connecting, and in some cases forming part of, the β-sheet structure. The CDRs in each chain are held together in close proximity by the FR regions and, with the CDRs from the other chain, contribute to the formation of the antigen binding site of antibodies. Kabat et al., supra. The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody-dependent cellular toxicity.

Papain digestion of antibodies produces two identical antigen binding fragments, called Fab fragments, each with a single antigen binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an $F(ab')_2$ fragment that has two antigen combining sites and is still capable of cross linking antigen.

"Fv" is the minimum antibody fragment which contains a complete antigen recognition and binding site. This region consists of a dimer of one heavy and one light chain variable domain in tight, non-covalent association. It is in this configuration that the three CDRs of each variable domain interact to define an antigen binding site on the surface of the V_H-V_L dimer. Collectively, the six CDRs confer antigen binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

A "Fab" fragment contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. $F(ab')_2$ antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other, chemical couplings of antibody fragments are also known.

The light chains of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (κ)and lambda (λ), based on the amino acid sequences of their constant domains.

Depending on the amino acid sequence of the constant domain of their heavy chains, immunoglobulins can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG and IgM, and several of these may be further divided into subclasses (isotypes), *e.g.* IgG-1, IgG-2, IgG-3, and IgG-4; IgA-1 and IgA-2. The heavy chain constant domains that correspond to the different classes of immunoglobulins are called α, delta, epsilon, γ, and, μ, respectively. The subunit structures and three-dimensional

configurations of different classes of immunoglobulins are well known.

The term "antibody" is used in the broadest sense and specifically covers single monoclonal antibodies (including agonist and antagonist antibodies), antibody compositions with polyepitopic specificity, as well as antibody fragments (*e.g.*, Fab, $F(ab^1)_2$, and Fv), so long as they exhibit the desired biological activity.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they are synthesized by the hybridoma culture, uncontaminated by other immunoglobulins. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler et al., *Nature* 256, 495 (1975), or may be made by recombinant DNA methods, see, *e.g.* U.S. Patent No. 4,816,567.

"Chimeric" antibodies (immunoglobulins) are antibodies wherein a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity. U.S. Patent No. 4,816,567.

"Humanized" forms of non-human (*e.g.* murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', $F(ab')_2$ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibody may comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and optimize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details see: Jones et al., *Nature* 321, 522 (1986); Reichmann et al., *Nature* 332, 323 (1988); and Presta, *Curr. Op. Struct. Biol.* 2, 593 (1992).

"Non-immunogenic in a human" means that upon contacting the humanized antibody in a therapeutically effective amount with appropriate tissue of a human, a state of sensitivity or resistance to the humanized antibody is not substantially demonstratable upon administration.

As used herein, "vascular endothelial cell growth factor," or "VEGF," refers to a mammalian growth factor as defined in U.S. Patent 5,332,671, including the human amino acid sequence of Fig. 1. The biological activity of native VEGF is shared by any analogue or variant thereof that is capable of promoting selective growth of vascular endothelial cells but not of bovine corneal endothelial cells, lens epithelial cells, adrenal cortex cells, BHK-21 fibroblasts, or keratinocytes, or that possesses an immune epitope that is immunologically cross-reactive with an antibody raised against at least one epitope of the corresponding native VEGF.

"Site-directed mutagenesis" is a technique standard in the art, and is conducted using a synthetic oligonucleotide primer complementary to a single-stranded phage DNA to be mutagenized except for limited mismatching, representing the desired mutation. Briefly, the synthetic oligonucleotide is used as a primer to direct synthesis of a strand complementary to the phage, and the resulting double-stranded DNA is transformed into a phage-supporting host bacterium. Cultures of the transformed bacteria are plated in top agar, permitting plaque formation from single cells that harbor the phage. Theoretically, 50% of the new plaques will contain the phage having, as a single strand, the mutated form; 50% will have the original sequence. The plaques are hybridized with kinased synthetic primer at a temperature that permits hybridization of an exact match, but at which the mismatches with the original strand are sufficient to prevent hybridization. Plaques that hybridize with the probe are then selected and cultured, and the DNA is recovered.

"Expression system" refers to DNA sequences containing a desired coding sequence and control sequences in operable linkage, so that hosts transformed with these sequences are capable of producing the encoded proteins. To effect transformation, the expression system may be included on a vector; however, the relevant DNA may then also be integrated into the host chromosome.

As used herein, "cell," "cell line," and "cell culture" are used interchangeably and all such designations include progeny. Thus, "transformants" or "transformed cells" includes the primary subject cell and cultures derived therefrom without regard for the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Mutant progeny that have the same functionality as screened for in the originally transformed cell are included. Where distinct designations are intended, it will be clear from the context.

"Plasmids" are designated by a lower case p preceded and/or followed by capital letters and/or numbers. The starting plasmids herein are commercially available, are publicly available on an unrestricted basis, or can be constructed from such available plasmids in accord with published procedures. In addition, other equivalent plasmids are known in the art and will be apparent to the ordinary artisan.

"Affinity binding" refers to the strength of the sum total of noncovalent interactions between a single antigen-binding site on an antibody and a single epitope. Low-affinity antibodies bind antigen weakly and tend to dissociate readily, whereas high-affinity antibodies bind antigen more tightly and remain bound longer.

"Transformation" means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integration. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described by Cohen, *Proc. Natl. Acad. Sci. USA* 69, 2110 (1972) and Mandel et al., *J. Mol. Biol.* 53, 154 (1970), is generally used for prokaryotes or other cells that contain substantial cell-wall barriers. For mammalian cells without such cell walls, the calcium phosphate precipitation method of Graham and van der Eb, *Virology* 52, 456 (1978) is preferred. General aspects of mammalian cell host system transformations have been described by Axel in U.S. Pat. No. 4,399,216 issued August 16, 1983. Transformations into yeast are typically carried out according to the method of Van Solingen et al., *J. Bact.* 130, 946 (1977) and Hsiao et al., *Proc. Natl. Acad. Sci. USA* 76, 3829 (1979). However, other methods for introducing DNA into cells such as by nuclear injection, electroporation or by protoplast fusion may also be used.

"Recovery" or "isolation" of a given fragment of DNA from a restriction digest means separation of the digest on polyacrylamide or agarose gel by electrophoresis, identification of the fragment of interest by comparison of its mobility versus that of marker DNA fragments of known molecular weight, removal of the gel section containing the desired fragment, and separation of the gel from DNA. This procedure is known generally. For example, see Lawn et al., *Nucleic Acids Res.* 9, 6103 (1981) and Goeddel et al., *Nucleic Acids Res.* 8, 4057 (1980).

"Ligation" refers to the process of forming phosphodiester bonds between two double stranded nucleic acid fragments. Unless otherwise provided, ligation may be accomplished using known buffers and conditions with 10 units of T4 DNA ligase ("ligase") per 0.5 mg of approximately equimolar amounts of the DNA fragments to be ligated.

The term "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, a ribosome binding site, and possibly, other as yet poorly understood sequences. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

Nucleic acid is "operably linked" or "operatively linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or a secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" or "operatively linked" means that the DNA sequences being linked are contiguous and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, then synthetic oligonucleotide adaptors or linkers are used in accord with conventional practice.

As used herein, "representatively numbered" refers to a position number of a residue in a particular sequence and corresponding position numbers in different sequences. Corresponding position numbers are those positions within sequences, generally human antibody framework sequences, which are functionally equivalent to the respresentatively numbered position when used in the construction of a humanized antibody.

Ordinarily, the terms "amino acid" and "amino acids" refer to all naturally occurring L-α-amino acids. In some embodiments, however, D-amino acids may be present in the polypeptides or peptides of the present invention in order to facilitate conformational restriction. The amino acids are identified by either the single-letter or three-letter designations:

	Asp	D	aspartic acid	Ile	I	isoleucine
	Thr	T	threonine	Leu	L	leucine
	Ser	S	serine	Tyr	Y	tyrosine
	Glu	E	glutamic acid	Phe	F	phenylalanine
5	Pro	P	proline	His	H	histidine
	Gly	G	glycine	Lys	K	lysine
	Ala	A	alanine	Arg	R	arginine
	Cys	C	cysteine	Trp	W	tryptophan
	Val	V	valine	Gln	Q	glutamine
10	Met	M	methionine	Asn	N	asparagine

The term "amino acid sequence variant" refers to molecules with some differences in their amino acid sequences as compared to a native amino acid sequence.

15 Substitutional variants are those that have at least one amino acid residue in a native sequence removed and a different amino acid inserted in its place at the same position. The substitutions may be single, where only one amino acid in the molecule has been substituted, or they may be multiple, where two or more amino acids have been substituted in the same molecule.

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Hybridization is preferably performed under "stringent conditions" which means (1) employing low ionic strength and high temperature for washing, for example, 0.015 sodium chloride/0.0015 M sodium citrate/0.1% sodium dodecyl sulfate at 50°C, or (2) employing during hybridization a denaturing agent, such as formamide, for example, 50% (vol/vol) 25 formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 nM sodium phosphate buffer at pH 6.5 with 750 mM sodium chloride, 75 mM sodium citrate at 42°C. Another example is use of 50% formamide, 5 x SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6/8), 0.1% sodium pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50 µg/ml), 0.1% SDS, and 10% dextran sulfate at 30 42°C, with washes at 42°C in 0.2 x SSC and 0.1% SDS. Yet another example is hybridization using a buffer of 10% dextran sulfate, 2 x SSC (sodium chloride/sodium citrate) and 50% formamide at 55°C, followed by a high-stringency wash consisting of 0.1 x SSC

containing EDTA at 55°C. When a nucleic acid sequence of a nucleic acid molecule is provided, other nucleic acid molecules hybridizing thereto under the conditions described above are considered within the scope of the sequence.

Where amino acid sequences are described it is understood that these sequences can be reproduced by reconstructing the amino acid sequence synthetically or by mutation. Alternatively, it is understood that recombinant techniques can be used such that the DNA encoding the amino acid sequences is recovered. The DNA is recovered by forming a library from the DNA encoding the desired amino acid sequences. Probes are then generated based on the amino acid sequences. DNA hybridizing to the probes is then isolated and analyzed to determine whether the product encoded by the DNA is the desired product. Generally, cells are transformed with the DNA (or RNA) and expression studies are performed.

B. General Methodology for Humanizing Antibodies

The methods described herein can be used to humanize any antibody. Similarly, it is understood that the humanized antibody specifically described herein, humanized anti-VEGF, can be reproduced by the methods described herein or by traditional DNA recombinant techniques. Specifically, since the critical framework residue mutations are described herein, the humanized antibody can be reproduced to have the same mutations without being reproduced using the monovalent phage display system. Rather, the DNA encoding the described amino acid sequences can be synthesized or reproduced by traditional DNA recombinant techniques. The DNA product can then be expressed, identified and recovered. Alternatively, site-directed mutagenesis can be performed on the antibody by methods known in the art, or the antibody can be synthesized so as to have the mutations described herein.

A particularly preferred method for producing the humanized antibodies described herein involves the following: preparing an antibody phagemid vector for monovalent display of Fab fragments having CDR sequences transplanted by site-directed mutagenesis onto a vector which codes for a human $V_L\kappa I$-$C\kappa_I$ light chain and human V_H III-C_H 1γ heavy chain Fd; constructing the antibody Fab phagemid library by random mutagenesis of a small set of selected critical framework residues; expressing and purifying the humanized Fab fragments; selecting humanized Fab variants; and, determining binding affinities. These steps do not

have to be performed in any particular order. These steps are specifically described below in the "specific example" but are generally performed as follows:

Preparation of antibody phagemid vector for monovalent display of Fab fragments

First an antibody to be humanized is selected and the complementary determining regions (CDRs) identified. The CDR sequences of the antibody can be identified according to the sequence definition of Kabat et al., supra. The CDR sequences are transplanted by site-directed mutagenesis onto a vector which codes for a human $V_L\kappa I$-$C\kappa_1$ light chain and human $V_H III$-$C_H 1\gamma_1$ heavy chain Fd . The Fab encoding sequence can then be subcloned into a phagemid vector. This construct encodes an initial humanized antibody wherein the C-terminus of the heavy chain is fused precisely to the carboxyl portion of a phage coat protein. Perferably, a phagemid vector is selected which provides expression of both secreted heavy chain or heavy chain-gene III fusions in *supE* suppressor strains of *E. coli*.

Construction of the antibody Fab phagemid library

Based on the cumulative results from humanizing a number of non-human antibodies onto a human $V_L\kappa I$-$V_H III$ framework, it was considered that framework changes required to optimize antigen binding are limited to some subset of the residues. See, Carter et al., *PNAS USA* 89, 4285 (1992); Presta et al., *J. Immunol.* 151, 2623 (1993); Eigenbrot et al., *Proteins* 18, 49-62 (1994); Shalaby et al., *J. Exp. Med.* 175, 217 (1992). Accordingly, a novel group of residues was selected for randomization. Randomizing these identified key framework residues provides the desired library of Fab variants to be displayed on the surface of filamentous phage. Specifically, V_L residues 4 and 71 and V_H residues 24, 37,67,69,71,71,75,76,78,93 and 94 have been selected as key framework residues important for antigen binding and targeted for randomization.

Expression and purification of humanized Fab fragments

Various methods are known in the art to express and purify fragments. As described herein, an *E. coli* strain 34B8, a nonsuppressor, was transformed with phagemid pMB419, or variants thereof. Single colonies were grown overnight at 37°C in 5 mL 2YT containing 50 µg/mL carbenicillin. These cultures were diluted into 200 mL AP5 medium, described in Chang et al., *Gene* 55, 189 (1987), containing 20 µg/mL carbenicillin and incubated for 26

hours at 30°C. The cells were pelleted at 4000 x g and frozen at -20°C for at least 2 hours. Cell pellets were then resuspended in 5 mL of 10 mM Tris-HCl (pH 7.6) containing 1 mM EDTA, shaken at 4°C for 90 minutes and centrifuged at 10,000 x g for 15 minutes. The supernatant was applied to a 1 mL streptococcal protein G-SEPHAROSE column (a column produced by Pharmacia) and washed with 10 mL of 10 mM MES (pH 5.5). The bound Fab fragment was eluted with 2.5 mL 100 mM acetic acid and immediately neutralized with 0.75 mL 1M TrisHCl, pH 8.0. Fab preparations were buffer-exchanged into PBS and concentrated using CENTRICON-30 concentrators (produced by Amicon). Typical yields of Fab were approximately 1 mg/L culture, post-protein G purification. Purified Fab samples were characterized by electrospray mass spectrometry, and concentrations were determined by amino acid analysis.

Selection of humanized Fab variants

Purified labeled antigen is coated onto a microtiter plate. The coating solution is discarded, the wells blocked, and phagemid stock is added. After a period, the wells are washed and the bound phage eluted and titered. The remaining phage eluted from the VEGF-coated well are propagated for use in the next selection cycle. This process can be repeated several times to obtain the desired number of clones. For example, a few dozen individual clones can be selected and sequenced.

Determination of VEGF binding affinities

Association and dissociation rate constants for binding of the humanized variants to VEGF are measured. Binding profiles are analyzed and those variants showing the highest affinities are selected.

Administration of the humanized anti-VEGF

Administration of the humanized anti-VEGF can be extrapolated from the data presented on the murine anti-VEGF described in Kim et al., *Growth Factors* 7, 53 (1992); Kim et al., *Nature* 362, 841 (1993). In particular, Kim et al. demonstrates that as little as 10 μg twice weekly of the VEGF antibody resulted in significant inhibition of tumor growth. Maximal effects were achieved with antibody doses of 50-100 μg.

The following example is intended merely to illustrate the best mode now known for practicing the invention but the invention is not to be considered as limited to the details of this example.

Specific Example I

Construction of the phagemid vector and the initial humanized anti-VEGF

The murine anti-VEGF mAb A4.6.1 has been previously described by Kim et al, *Growth Factors*, 7, 53 (1992); Kim et al., *Nature*, 362, 841 (1993). The first Fab variant of humanized A4.6.1, hu2.0, was constructed by site-directed mutagenesis using a deoxyuridine-containing template of plasmid pAK2 which codes for a human $V_L\kappa I$-$C\kappa_1$ light chain and human $V_H III$-$C_H 1\gamma_1$ heavy chain Fd fragment. Carter et al., *PNAS USA* 89, 4285 (1992). The transplanted A4.6.1 CDR sequences were chosen according to the sequence definition of Kabat et al., Sequences of Proteins of Immunological Interest (5th), Public Health Service, National Institutes of Health, Bethesda, MD. (1991), except for CDR-H1 which we extended to encompass both sequence and structural definitions, viz V_H residues 26-35, Chothia et al., *J. Mol. Biol.* 196, 901 (1987). The Fab encoding sequence was subcloned into the phagemid vector phGHamg3. Bass and Wells, *Proteins*, 8, 309 (1990); Lowman et al., *Biochem.* 30, 10832 (1991). This construct, pMB4-19, encodes the initial humanized A4.6.1 Fab, hu2.0, with the C-terminus of the heavy chain fused precisely to the carboxyl portion of the M13 gene III coat protein. pMB4-19 is similar in construction to pDH188, a previously described plasmid for monovalent display of Fab fragments. Garrard et al., *Biotechn.* 9: 1373-1377 (1991). Notable differences between pMB4-19 and pDH188 include a shorter M13 gene III segment (codons 249-406) and use of an amber stop codon immediately following the antibody heavy chain Fd fragment. This permits expression of both secreted heavy chain or heavy chain-gene III fusions in *supE* suppressor strains of *E. coli*.

The initial humanized A4.6.1 Fab fragment (hu2.0) in which the CDRs from A4.6.1 were grafted onto a human $V_{L\kappa}I$-$V_H III$ framework is shown in Figure 1. The V_L domain of hu2.0 is set forth in SEQ ID NO: 7 and the V_H domain of hu2.0 is set forth in SEQ ID NO: 10.

All residues other than the grafted CDRs were maintained as the human sequence. Binding

of this initial humanized antibody to VEGF was so weak as to be undetectable. Based on the relative affinity of other weakly-binding humanized A4.6.1 variants (data not shown), the K_D for binding of hu2.0 was estimated at >7 μM. This contrasts with an affinity of 1.6 nM for a chimeric Fab construct consisting of the intact V_L and V_H domains from murine A4.6.1 and human constant domains. Thus, binding of hu2.0 to VEGF was at least 4000-fold reduced relative to the chimera.

Design of the anti-VEGF Fab phagemid library

The group of framework changes required to optimize antigen binding when using human $V_L\kappa I$-$V_H III$ framework were selected as shown in Table 1 and Figure 2. The humanized A4.6.1 phagemid library was constructed by site-directed mutagenesis according to the method of Kunkel et al., *Methods Enzymol.* 204, 125 (1991). A derivative of pMB4-19 containing TAA stop triplets at V_H codons 24, 37, 67 and 93 was prepared for use as the mutagenesis template (all sequence numbering according to Kabat et al., supra. This modification was to prevent subsequent background contamination by wild type sequences. The codons targeted for randomization were 4 and 71 (light chain) and 24, 37, 67, 69, 71, 73, 75, 76, 78, 93 and 94 (heavy chain).

Table 1: Key framework residues important for antigen binding and targeted for randomization

Framework residue		Human $V\kappa_L I$, $V_H III$ consensus residue	Murine A4.6.1 residue	Randomization[a]
V_L:	4	Met	Met	Met,Leu
	71	Phe	Tyr	Phe, Tyr
V_H:	24	Ala	Ala	Ala, Val, Thr
	37	Val	Val	Val, Ile
	67	Phe	Phe	Phe, Val, Thr, Leu, Ile, Ala
	69	Ile	Phe	Ile, Phe
	71	Arg	Leu	Arg[b], Leu[b]
	73	Asp	Thr	Asp[b], Thr[b]
	75	Lys	Ala	Lys[b], Ala[b]
	76	Asn	Ser	Asn[b], Ser[b]
	78	Leu	Ala	Leu, Ala, Val, Phe
	93	Ala	Ala	Ala, Val, Leu, Ser, Thr
	94	Arg	Lys	Arg, Lys

[a] Amino acid diversity in phagemid library
[b] V_H 71, 73, 75, 76 randomized to yield the all-murine (L71/T73/A75/S76) or all-human (R71/D73/K75/N76) $V_H III$ tetrad

A concern in designing the humanized A4.6.1 phagemid library was that residues targeted for randomization were widely distributed across the V_L and V_H sequences. Limitations in the length of synthetic oligonucleotides requires that simultaneous randomization of each of these framework positions can only be achieved through the use of multiple oligonucleotides. However, as the total number of oligonucleotides increases, the efficiency of mutagenesis decreases (*i.e.* the proportion of mutants obtained which incorporate sequence derived from all of the mutagenic oligonucleotides). To circumvent this problem, two features were incorporated into the library construction. The first was to prepare four different mutagenesis templates coding for each of the possible V_L framework combinations. This was simple to do given the limited diversity of the light chain framework (only 4 different sequences), but was beneficial in that it eliminated the need for two oligonucleotides from the mutagenesis strategy. Secondly, two 126 base oligonucleotides were preassembled from smaller synthetic fragments. This made possible randomization of V_H codons 67, 69, 71, 73,

75, 76, 93 and 94 with a single long oligonucleotide, rather than two smaller ones. The final randomization mutagenesis strategy therefore employed only two oligonucleotides simultaneously onto four different templates.

More specifically, in order to randomize heavy chain codons 67, 69, 71, 73, 75, 76, 78, 93 and 94 with a single mutagenic oligonucleotide, two 126-mer oligonucleotides were first preassembled from 60 and 66-mer fragments by template-assisted enzymatic ligation. Specifically, 1.5 nmol of 5' phosphorylated oligonucleotide GAT TTC AAA CGT CGT NYT ACT WTT TCT AGA GAC AAC TCC AAA AAC ACA BYT TAC CTG CAG ATG AAC (SEQ ID NO: 12) or GAT TTC AAA CGT CGT NYT ACT WTT TCT TTA GAC ACC TCC GCA AGC ACA BYT TAC CTG CAG ATG AAC (SEQ ID NO: 1) were combined with 1.5 nmol of AGC CTG CGC GCT GAG GAC ACT GCC GTC TAT TAC TGT DYA ARG TAC CCC CAC TAT TAT GGG (SEQ ID NO: 2). The randomized codons are underlined and N represents A/G/T/C; W represents A/T; B represents G/T/C; D represents G/A/T; R represents A/G; and Y represents C/T ("/" represents "or"). Then, 1.5 nmol of template oligonucleotide CTC AGC GCG CAG GCT GTT CAT CTG CAG GTA (SEQ ID NO: 3), with complementary sequence to the 5' ends of SEQ ID NOS: 12 and 1 and the 3' end of SEQ ID NO: 3 was added to hybridize to each end of the ligation junction. To this mixture, *Taq* ligase (thermostable ligase from New England Biolabs) and buffer were added, and the reaction mixture was subjected to 40 rounds of thermal cycling, (95°C for 1.25 minutes and 50°C for 5 minutes) so as to cycle the template oligonucleotide between ligated and unligated junctions. The product 126-mer oligonucleotides were purified on a 6% urea/TBE polyacrylamide gel and extracted from the polyacrylamide in buffer. The two 126-mer products were combined in equal ratio, ethanol precipitated and finally solubilized in 10 mM Tris-HCl, 1 mM EDTA. The mixed 126-mer oligonucleotide product was labeled 504-01.

Randomization of select framework codons (V_L 4, 71; V_H 24, 37, 67, 69, 71, 73, 75, 76, 93, 94) was thus effected in two steps. First, V_L randomization was achieved by preparing three additional derivatives of the modified pMB4-19 template. Framework codons 4 and 71 in the light chain were replaced individually or pairwise using the two mutagenic oligonucleotides GCT GAT ATC CAG TTG ACC CAG TCC CCG (SEQ ID NO: 13) and

TCT GGG ACG GAT TAC ACT CTG ACC ATC (SEQ ID NO: 4). Deoxyuridine containing template was prepared from each of these new derivatives. Together with the original template, these four constructs coded for each of the four possible light chain framework sequence combinations (see Table 1).

Oligonucleotides 504-01, the mixture of two 126-mer oligonucleotides, and CGT TTG TCC TGT GCA RYT TCT GGC TAT ACC TTC ACC AAC TAT GGT ATG AAC TGG RTC CGT CAG GCC CCG GGT AAG (SEQ ID NO: 5) were used to randomize heavy chain framework codons using each of the four templates just described. The four libraries were electroporated into *E. coli* XL-1 BLUE CELLS (marker cells produced by Stratagene) and combined. The total number of independent transformants was estimated at $>1.2 \times 10^8$, approximately 1,500-fold greater than the maximum number of DNA sequences in the library.

From this strategy, each of residues 4 and 71 in the light chain and 24, 37, 67, 78 and 93 from the heavy chain were partially randomized to allow the selection of either the murine A4.6.1, human $V_L\kappa I$-$V_H III$ sequence, or sequences commonly found in other human and murine frameworks (Table I). Note that randomization of these residues was not confined to a choice between the human $V_L\kappa I$-$V_H III$ consensus or A4.6.1 framework sequences. Rather, inclusion of additional amino acids commonly found in other human and murine framework sequences allows for the possibility that additional diversity may lead to the selection of tighter binding variants.

Some of the heavy chain framework residues were randomized in a binary fashion according to the human $V_H III$ and murine A4.6.1 framework sequences. Residues V_H 71, 73, 75 and 76 are positioned in a hairpin loop adjacent to the antigen binding site. The side chains of V_H 71 and 73 are largely buried in canonical antibody structures and their potential role in shaping the conformation of CDR-H2 and CDR-H3 is well known. Kettleborough et al., *Protein Eng.* 4, 773 (1991); Carter et al., *PNAS USA* 89, 4285 (1992); Shalaby et al., *J. Exp. Med.* 175, 217 (1992). On the other hand, although the side chains of V_H 75 and 76 are solvent exposed (Figure 2), it has nevertheless been observed that these two residues can also influence antigen binding (Eigenbrot, *Proteins* 18, 49 [1994]), presumably due to direct antigen contact in some antibody-antigen complexes. Because of their proximity in sequence

and possible interdependence, V_H 71, 73, 75 and 76 were randomized en bloc such that only two possible combinations of this tetrad could be selected; either all human V_HIII or all murine A4.6.1 sequence. Finally, V_H residues 69 and 94 were randomized, but only to represent the V_HIII and A4.6.1 sequences. The V_H 69 and 94 were not replaced in previous antibody humanizations, but because they differ between the V_HIII consensus and A4.6.1 sequences (Figure 1) and have been noted as potentially important for proper CDR conformation (Foote et al., *J. Mol. Biol.* 224, 487 [1992]), they were included in this randomization strategy.

Humanized A4.6.1 Fab library displayed on the surface of phagemid

A variety of systems have been developed for the functional display of antibody fragments on the surface of filamentous phage. Winter et al., *Ann. Rev. Immunol.* 12, 433 (1994). These include the display of Fab or single chain Fv (scFv) fragments as fusions to either the gene III or gene VIII coat proteins of M13 bacteriophage. The system selected herein is similar to that described by Garrard et al., *Biotechn.* 9, 1373 (1991) in which a Fab fragment is monovalently displayed as a gene III fusion (Figure 3). This system has two notable features. In particular, unlike scFvs, Fab fragments have no tendency to form dimeric species, the presence of which can prevent selection of the tightest binders due to avidity effects. Additionally, the monovalency of the displayed protein eliminates a second potential source of avidity effects that would otherwise result from the presence of multiple copies of a protein on each phagemid particle. Bass and Wells, *Proteins* 8, 309 (1990); Lowman et al., *Biochemistry* 30, 10832 (1991).

Phagemid particles displaying the humanized A4.6.1 Fab fragments were propagated in *E. coli* XL-1 Blue cells. Briefly, cells harboring the randomized pMB4-19 construct were grown overnight at 37°C in 25 mL 2YT medium containing 50 μg/mL carbenicillin and approximately 10^{10} M13KO7 helper phage (Viera and Messing, *Methods Enzymol.* 153, 3 [1987]). Phagemid stocks were purified from culture supernatants by precipitation with a saline polyethylene glycol solution, and resuspended in 100 μL PBS (approximately 10^{14} phagemid/mL).

Selection of humanized A4.6.1 Fab variants

Purified $VEGF_{121}$ (100 µL at 10 µg/mL in PBS) was coated onto a microtiter plate well overnight at 4°C. The coating solution was discarded and this well and an uncoated well were blocked with 6% skim milk for 1 hour and washed with PBS containing 0.05% TWEEN-20 (detergent). Then, 10 µL of phagemid stock, diluted to 100 µL with 20 mM Tris (pH 7.5) containing 0.1% BSA and 0.05% TWEEN-20, was added to each well. After 2 hours, the wells were washed and the bound phage eluted with 100 µL of 0.1 M glycine (pH 2.0), and neutralized with 25 µL of 1M Tris pH 8.0. An aliquot of this was used to titer the number of phage eluted. The remaining phage eluted from the VEGF-coated well were propagated for use in the next selection cycle. A total of 8 rounds of selection was performed after which time 20 individual clones were selected and sequenced (Sanger et al., *PNAS USA* 74, 5463 [1977]).

Variants from the humanized A4.6.1 Fab phagemid library were thusly selected based on binding to VEGF. Enrichment of functional phagemid, as measured by comparing titers for phage eluted from a VEGF-coated versus uncoated microtiter plate well, increased up to the seventh round of affinity panning. After one additional round of sorting, 20 clones were sequenced to identify preferred framework residues selected at each position randomized. These results, summarized in Table 2, revealed strong consensus amongst the clones selected. Ten out of the twenty clones had the identical DNA sequence, designated hu2.10. Of the thirteen framework positions randomized, eight substitutions were selected in hu2.10 (V_L 71; V_H 37, 71, 73, 75, 76, 78 and 94). Interestingly, residues VH 37 (Ile) and 78 (Val) were selected neither as the human V_HIII or murine A4.6.1 sequence. This result suggests that some framework positions may benefit from extending the diversity beyond the target human and parent murine framework sequences.

Table 2: Sequences selected from the humanized A4.6.1 phagemid Fab library

Variant	Residue substitutions V_L		V_H										
	4	71	24	37	67	69	71	73	75	76	78	93	94
murine A4.6.1	M	Y	A	V	F	F	L	T	A	S	A	A	K
hu2.0 (CDR-graft)	M	F	A	V	F	I	R	N	K	N	L	A	R
Phage-selected clones:													
hu2.1 (2)	-	Y	-	I	-	-	-	-	-	-	V	-	K
hu2.2 (2)	L	Y	-	I	-	-	-	-	-	-	V	-	K
hu2.6 (1)	L	-	-	I	T	-	L	T	A	S	V	-	K
hu2.7 (1)	L	-	-	I	T	-	-	-	-	-	V	-	K
hu2.10 (10)	-	Y	-	I	-	-	L	T	A	S	V	-	K

Differences between hu2.0 and murine A4.6.1 antibodies are underlined. The number of identical clones identified for each phage-selected sequence is indicated in parentheses. Dashes in the sequences of phage-selected clones indicate selection of the human $V_L\kappa I$-$V_H III$ framework sequence (i.e. as in hu2.0).

There were four other unique amino acid sequences among the remaining ten clones analyzed: hu2.1, hu2.2, hu2.6 and hu2.7. All of these clones, in addition to hu2.10, contained identical framework substitutions at positions V_H 37 (Ile), 78 (Val) and 94 (Lys), but retained the human $V_H III$ consensus sequence at positions 24 and 93. Four clones had lost the light chain coding sequence and did not bind VEGF when tested in a phage ELISA assay (Cunningham et al., *EMBO J.* 13, 2508 [1994]). We have occasionally noted the loss of heavy or light chain sequence with other Fab phagemid libraries (unpublished data), and these clones are presumably selected for on the basis of enhanced expression. Such artifacts can often be minimized by reducing the number of sorting cycles or by propagating libraries on solid media.

Determination of VEGF binding affinities

Association (k_{on}) and dissociation (k_{off}) rate constants for binding of humanized A4.6.1 Fab variants to $VEGF_{121}$ were measured by surface plasmon resonance (Karlsson et al, *J. Immun. Methods* 145, 229 [1991]) on a Pharmacia BIAcore instrument. $VEGF_{121}$ was covalently immobilized on the biosensor chip via primary amino groups. Binding of humanized A4.6.1 Fab variants was measured by flowing solutions of Fab in PBS/0.05% TWEEN-20 (detergent) over the chip at a flow rate of 20 µL/min. Following each binding measurement, residual Fab was stripped from the immobilized ligand by washing with 5 µL of 50 mM aqueous HCl at 3 µL/min. Binding profiles were analyzed by nonlinear regression using a simple monovalent binding model (BIAevaluation software v2.0; Pharmacia).

Phage-selected variants hu2.1, hu2.2, hu2.6, hu2.7 and hu2.10 were expressed in *E. coli* using shake flasks and Fab fragments were purified from periplasmic extracts by protein G affinity chromatography. Recovered yields of Fab for these five clones ranged from 0.2 (hu2.6) to 1.7 mg/L (hu2.1). The affinity of each of these variants for antigen (VEGF) measured by surface plasmon resonance on a BIAcore instrument as shown in Table 3.

Table 3: VEGF binding affinity of humanized A4.6.1 Fab variants.

Variant	k_{on}	k_{off}	K_D	K_D (A4.6.1) / K_D (mut)	
	$M^{-1}s^{-1}/10^4$	$10^4 s^{-1}$	nM		
A4.6.1 chimera	5.4	0.85	1.6		
hu2.0	ND	ND	>7000**	>4000	
Phage selected clones:					
hu2.1	0.70	18	260	170	
hu2.2	0.47	16	340	210	
hu2.6	0.67	4.5	67	40	
hu2.7	0.67	24	360	230	
hu2.10	0.63	3.5	55	35	
*hu2.10V	2.0	1.8	9.3	5.8	

*hu2.10V = hu2.10 with mutation V_L Leu46 -> Val; Estimated errors in the Biacore binding measurements are +/- 25%; **Too weak to measure, estimate of lower bound

Analysis of this binding data revealed that the consensus clone hu2.10 possessed the highest affinity for VEGF out of the five variants tested. Thus our Fab phagemid library was selectively enriched for the tightest binding clone. The calculated K_D for hu2.10 was 55 nM, at least 125-fold tighter than for hu2.0- which contains no framework changes (K_D >7 μM). The other four selected variants all exhibited weaker binding to VEGF, ranging down to a K_D of 360 nM for the weakest (hu2.7). Interestingly, the K_D for hu2.6, 67 nM, was only marginally weaker than that of hu2.10 and yet only one copy of this clone was found among 20 clones sequenced. This may have due to a lower level of expression and display, as was the case when expressing the soluble Fab of this variant. However, despite the lower expression rate, this variant is useful as a humanized antibody.

Additional improvement of humanized variant hu2.10

Despite the large improvement in antigen affinity over the initial humanized variant, binding of hu2.10 to VEGF was still 35-fold weaker than a chimeric Fab fragment containing the murine A4.6.1 V_L and V_H domains. This considerable difference suggested that further optimization of the humanized framework might be possible through additional mutations. Of the vernier residues identified by Foote et al., *J. Mol. Biol.* 196, 901 (1992), only residues V_L 46, V_H 2 and V_H 48 differed in the A4.6.1 versus human V_κI-V_HIII framework (Figure 1) but were not randomized in our phagemid library. A molecular model of the humanized A4.6.1 Fv fragment showed that V_L 46 sits at the V_L-V_H interface and could influence the conformation of CDRH3. Furthermore, this amino acid is almost always leucine in most $V_L\kappa$ frameworks (Kabat et al., supra.), but is valine in A4.6.1. Accordingly, a Leu -> Val substitution was made at this position in the background of hu2.10. Analysis of binding kinetics for this new variant, hu2.10V, indicated a further 6-fold improvement in the K_D for VEGF binding. The K_D for hu2.10V (9.3 nM) was thus within 6-fold that of the chimera. In contrast to V_L 46, no improvement in the binding affinity of hu2.10 was observed for replacement of either V_H 2 or V_H 48 with the corresponding residue from murine A4.6.1.

Interestingly, part of the improvement prior to the last change in affinity was due to an increase in the association rate constant (k_{on}), suggesting that V_L 46 may play a role in preorganizing the antibody structure into a conformation more suitable for antigen binding. Other mutations which affected antigen affinity were primarily due to changes in the

dissociation rate constant (k_{off}) for binding. Comparison of hu2.1 and hu2.10 reveals a 5-fold improvement in affinity for substitution of V_H residues 71, 73, 75, 76 with the A4.6.1 sequence. Conversion of V_L- 71 to the A4.6.1 sequence (Phe -> Tyr) had negligible effect on binding (hu2.2 vs hu2.7), while variants with leucine at V_L 4 bound marginally worse (<2-fold) than those with methionine, the naturally occurring residue in both the A4.6.1 and human V_{KL}I frameworks (hu2.2 vs hu2.1). Comparison of other humanized A4.6.1 variants not shown here revealed that the V_H 94 Arg -> Lys change resulted in a 5-fold improvement in K_D, either due to direct antigen contact by this residue, or to a structural role in maintaining the proper conformation of CDR-H3. Variant hu2.6 has three sequence differences relative to the consensus clone hu2.10, but nevertheless has a similar K_D, thereby suggesting that these three substitutions have little effect on antigen binding. The negligible effect of conservative changes at V_L 4 and 71 concurs with binding data for other variants, yet the change at V_H 67 (Phe -> Thr) had little effect on binding.

Concluding Remarks

The foregoing description details specific methods which can be employed to practice the present invention. Having detailed such specific methods, those skilled in the art will well enough know how to devise alternative reliable methods at arriving at the same information by using the fruits of the present invention. Thus, however detailed the foregoing may appear in text, it should not be construed as limiting the overall scope thereof; rather, the ambit of the present invention is to be determined only by the lawful construction of the appended claims. All documents cited herein are hereby expressly incorporated by reference.

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
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(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GATTTCAAAC GTCGTNYTAC TWTTTCTTTA GACACCTCCG CAAGCACABY TTACCTGCAG 60

ATGAAC 66

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

AGCCTGCGCG CTGAGGACAC TGCCGTCTAT TACTGTDYAA RGTACCCCCA CTATTATGGG 60

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CTCAGCGCGC AGGCTGTTCA TCTGCAGGTA 30

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TCTGGGACGG ATTACACTCT GACCATC 27

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 75 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CGTTTGTCCT GTGCARYTTC TGGCTATACC TTCACCAACT ATGGTATGAA CTGGRTCCGT 60

CAGGCCCCGG GTAAG 75

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 107 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
Asp Ile Gln Met Thr Gln Thr Thr Ser Ser Leu Ser Ala Ser Leu Gly
1               5                  10                  15

Asp Arg Val Ile Ile Ser Cys Ser Ala Ser Gln Asp Ile Ser Asn Tyr
            20                  25                  30

Leu Asn Trp Tyr Gln Gln Lys Pro Asp Gly Thr Val Lys Val Leu Ile
        35                  40                  45

Tyr Phe Thr Ser Ser Leu His Ser Gly Val Pro Ser Arg Phe Ser Gly
    50                  55                  60

Ser Gly Ser Gly Thr Asp Tyr Ser Leu Thr Ile Ser Asn Leu Glu Pro
65                  70                  75                  80

Glu Asp Ile Ala Thr Tyr Tyr Cys Gln Gln Tyr Ser Thr Val Pro Trp
                85                  90                  95

Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys
            100                 105
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 107 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1               5                  10                  15

Asp Arg Val Thr Ile Thr Cys Ser Ala Ser Gln Asp Ile Ser Asn Tyr
            20                  25                  30

Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
        35                  40                  45

Tyr Phe Thr Ser Ser Leu His Ser Gly Val Pro Ser Arg Phe Ser Gly
    50                  55                  60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
65                  70                  75                  80

Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Tyr Ser Thr Val Pro Trp
                85                  90                  95
```

Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys
100 105

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 107 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1 5 10 15

Asp Arg Val Thr Ile Thr Cys Ser Ala Ser Gln Asp Ile Ser Asn Tyr
20 25 30

Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
35 40 45

Tyr Phe Thr Ser Ser Leu His Ser Gly Val Pro Ser Arg Phe Ser Gly
50 55 60

Ser Gly Ser Gly Thr Asp Tyr Thr Leu Thr Ile Ser Ser Leu Gln Pro
65 70 75 80

Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Tyr Ser Thr Val Pro Trp
85 90 95

Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys
100 105

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 123 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Glu Ile Gln Leu Val Gln Ser Gly Pro Glu Leu Lys Gln Pro Gly Glu
1 5 10 15

Thr Val Arg Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Asn Tyr
20 25 30

Gly Met Asn Trp Val Lys Gln Ala Pro Gly Lys Gly Leu Lys Trp Met
35 40 45

Gly Trp Ile Asn Thr Tyr Thr Gly Glu Pro Thr Tyr Ala Ala Asp Phe
50 55 60

Lys Arg Arg Phe Thr Phe Ser Leu Glu Thr Ser Ala Ser Thr Ala Tyr
65 70 75 80

Leu Gln Ile Ser Asn Leu Lys Asn Asp Asp Thr Ala Thr Tyr Phe Cys
85 90 95

Ala Lys Tyr Pro His Tyr Tyr Gly Ser Ser His Trp Tyr Phe Asp Val
100 105 110

Trp Gly Ala Gly Thr Thr Val Thr Val Ser Ser
115 120

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 123 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1 5 10 15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr Asn Tyr
20 25 30

Gly Met Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
35 40 45

Gly Trp Ile Asn Thr Tyr Thr Gly Glu Pro Thr Tyr Ala Ala Asp Phe
50 55 60

Lys Arg Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr
65 70 75 80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
85 90 95

Ala Arg Tyr Pro His Tyr Tyr Gly Ser Ser His Trp Tyr Phe Asp Val
100 105 110

Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
115 120

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 123 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1 5 10 15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr Asn Tyr
20 25 30

Gly Met Asn Trp Ile Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
35 40 45

Gly Trp Ile Asn Thr Tyr Thr Gly Glu Pro Thr Tyr Ala Ala Asp Phe
50 55 60

Lys Arg Arg Phe Thr Ile Ser Leu Asp Thr Ser Ala Ser Thr Val Tyr
65 70 75 80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
85 90 95

Ala Lys Tyr Pro His Tyr Tyr Gly Ser Ser His Trp Tyr Phe Asp Val
100 105 110

Trp Gly Gln Gly Thr Ser Val Thr Val Ser Ser
115 120

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 66 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GATTTCAAAC GTCGTNYTAC TWTTTCTAGA GACAACTCCA AAAACACABY TTACCTGCAG 60

ATGAAC 66

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GCTGATATCC AGTTGACCCA GTCCCCG 27

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6072 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 459..460

(D) OTHER INFORMATION: /note= "Light chain begins at base no. 459."

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1101..1102
(D) OTHER INFORMATION: /note= "Light chain terminates at base no. 1101."

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1254..1255
(D) OTHER INFORMATION: /note= "Heavy chain begins at base no. 1254."

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 2424..2425
(D) OTHER INFORMATION: /note= "Heavy chain terminates at base no. 2424."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GAATTCAACT TCTCCATACT TTGGATAAGG AAATACAGAC ATGAAAAATC TCATTGCTGA 60

GTTGTTATTT AAGCTTTGGA GATTATCGTC ACTGCAATGC TTCGCAATAT GGCGCAAAAT 120

GACCAACAGC GGTTGATTGA TCAGGTAGAG GGGGCGCTGT ACGAGGTAAA GCCCGATGCC 180

AGCATTCCTG ACGACGATAC GGAGCTGCTG CGCGATTACG TAAAGAAGTT ATTGAAGCAT 240

CCTCGTCAGT AAAAAGTTAA TCTTTTCAAC AGCTGTCATA AAGTTGTCAC GGCCGAGACT 300

TATAGTCGCT TTGTTTTTAT TTTTTAATGT ATTTGTAACT AGAATTCGAG CTCGGTACCC 360

GGGGATCCTC TAGAGGTTGA GGTGATTTTA TGAAAAAGAA TATCGCATTT CTTCTTGCAT 420

CTATGTTCGT TTTTTCTATT GCTACAAACG CGTACGCTGA TATCCAGATG ACCCAGTCCC 480

CGAGCTCCCT GTCCGCCTCT GTGGGCGATA GGGTCACCAT CACCTGCAGC GCAAGTCAGG 540

ATATTAGCAA CTATTTAAAC TGGTATCAAC AGAAACCAGG AAAAGCTCCG AAAGTACTGA 600

TTTACTTCAC CTCCTCTCTC CACTCTGGAG TCCCTTCTCG CTTCTCTGGA TCCGGTTCTG 660

GGACGGATTA CACTCTGACC ATCAGCAGTC TGCAGCCAGA AGACTTCGCA ACTTATTACT 720

GTCAACAGTA TAGCACCGTG CCGTGGACGT TTGGACAGGG TACCAAGGTG GAGATCAAAC 780

GAACTGTGGC TGCACCATCT GTCTTCATCT TCCCGCCATC TGATGAGCAG TTGAAATCTG 840

GAACTGCTTC TGTTGTGTGC CTGCTGAATA ACTTCTATCC CAGAGAGGCC AAAGTACAGT 900

GGAAGGTGGA TAACGCCCTC CAATCGGGTA ACTCCCAGGA GAGTGTCACA GAGCAGGACA 960

GCAAGGACAG CACCTACAGC CTCAGCAGCA CCCTGACGCT GAGCAAAGCA GACTACGAGA 1020

AACACAAAGT CTACGCCTGC GAAGTCACCC ATCAGGGCCT GAGCTCGCCC GTCACAAAGA 1080

GCTTCAACAG GGGAGAGTGT TAAGCTGATC CTCTACGCCG GACGCATCGT GGCCCTAGTA 1140

CGCAACTAGT CGTAAAAAGG GTATCTAGAG GTTGAGGTGA TTTTATGAAA AAGAATATCG 1200

CATTTCTTCT TGCATCTATG TTCGTTTTTT CTATTGCTAC AAACGCGTAC GCTGAGGTTC 1260

AGCTGGTGGA GTCTGGCGGT GGCCTGGTGC AGCCAGGGGG CTCACTCCGT TTGTCCTGTG 1320

CAGCTTCTGG CTATACCTTC ACCAACTATG GTATGAACTG GATCCGTCAG GCCCCGGGTA 1380

AGGGCCTGGA ATGGGTTGGA TGGATTAACA CCTATACCGG TGAACCGACC TATGCTGCGG 1440

ATTTCAAACG TCGTTTTACT ATTTCTTTAG ACACCTCCGC AAGCACAGTT TACCTGCAGA 1500

TGAACAGCCT GCGCGCTGAG GACACTGCCG TCTATTACTG TGCAAAGTAC CCCCACTATT 1560

ATGGGAGCAG CCACTGGTAT TTCGACGTCT GGGGTCAAGG AACCCTGGTC ACCGTCTCCT 1620

CGGCCTCCAC CAAGGGCCCA TCGGTCTTCC CCCTGGCACC CTCCTCCAAG AGCACCTCTG 1680

GGGGCACAGC GGCCCTGGGC TGCCTGGTCA AGGACTACTT CCCCGAACCG GTGACGGTGT 1740

CGTGGAACTC AGGCGCCCTG ACCAGCGGCG TGCACACCTT CCCGGCTGTC CTACAGTCCT 1800

CAGGACTCTA CTCCCTCAGC AGCGTGGTGA CCGTGCCCTC CAGCAGCTTG GGCACCCAGA 1860

CCTACATCTG CAACGTGAAT CACAAGCCCA GCAACACCAA GGTCGACAAG AAAGTTGAGC 1920

CCAAATCTTG TGACAAAACT CACCTCTAGA GTGGCGGTGG CTCTGGTTCC GGTGATTTTG 1980

ATTATGAAAA GATGGCAAAC GCTAATAAGG GGGCTATGAC CGAAAATGCC GATGAAAACG 2040

CGCTACAGTC TGACGCTAAA GGCAAACTTG ATTCTGTCGC TACTGATTAC GGTGCTGCTA 2100

TCGATGGTTT CATTGGTGAC GTTTCCGGCC TTGCTAATGG TAATGGTGCT ACTGGTGATT 2160

TTGCTGGCTC TAATTCCCAA ATGGCTCAAG TCGGTGACGG TGATAATTCA CCTTTAATGA 2220

ATAATTTCCG TCAATATTTA CCTTCCCTCC CTCAATCGGT TGAATGTCGC CCTTTTGTCT 2280

TTAGCGCTGG TAAACCATAT GAATTTTCTA TTGATTGTGA CAAAATAAAC TTATTCCGTG 2340

GTGTCTTTGC GTTTCTTTTA TATGTTGCCA CCTTTATGTA TGTATTTTCT ACGTTTGCTA 2400

ACATACTGCG TAATAAGGAG TCTTAATCAT GCCAGTTCTT TTGGCTAGCG CCGCCCTATA 2460

CCTTGTCTGC CTCCCCGCGT TGCGTCGCGG TGCATGGAGC CGGGCCACCT CGACCTGAAT 2520

GGAAGCCGGC GGCACCTCGC TAACGGATTC ACCACTCCAA GAATTGGAGC CAATCAATTC 2580

TTGCGGAGAA CTGTGAATGC GCAAACCAAC CCTTGGCAGA ACATATCCAT CGCGTCCGCC 2640

ATCTCCAGCA GCCGCACGCG GCGCATCTCG GGCAGCGTTG GGTCCTGGCC ACGGGTGCGC 2700

ATGATCGTGC TCCTGTCGTT GAGGACCCGG CTAGGCTGGC GGGGTTGCCT TACTGGTTAG 2760

CAGAATGAAT CACCGATACG CGAGCGAACG TGAAGCGACT GCTGCTGCAA AACGTCTGCG 2820

ACCTGAGCAA CAACATGAAT GGTCTTCGGT TTCCGTGTTT CGTAAAGTCT GGAAACGCGG 2880

AAGTCAGCGC CCTGCACCAT TATGTTCCGG ATCTGCATCG CAGGATGCTG CTGGCTACCC 2940

TGTGGAACAC CTACATCTGT ATTAACGAAG CGCTGGCATT GACCCTGAGT GATTTTTCTC 3000

TGGTCCCGCC GCATCCATAC CGCCAGTTGT TTACCCTCAC AACGTTCCAG TAACCGGGCA 3060

TGTTCATCAT CAGTAACCCG TATCGTGAGC ATCCTCTCTC GTTTCATCGG TATCATTACC 3120

CCCATGAACA GAAATTCCCC CTTACACGGA GGCATCAAGT GACCAAACAG GAAAAAACCG 3180

CCCTTAACAT GGCCCGCTTT ATCAGAAGCC AGACATTAAC GCTTCTGGAG AAACTCAACG 3240

AGCTGGACGC GGATGAACAG GCAGACATCT GTGAATCGCT TCACGACCAC GCTGATGAGC 3300

TTTACCGCAG GATCCGGAAA TTGTAAACGT TAATATTTTG TTAAAATTCG CGTTAAATTT 3360

TTGTTAAATC AGCTCATTTT TTAACCAATA GGCCGAAATC GGCAAAATCC CTTATAAATC 3420

AAAAGAATAG ACCGAGATAG GGTTGAGTGT TGTTCCAGTT TGGAACAAGA GTCCACTATT 3480

AAAGAACGTG GACTCCAACG TCAAAGGGCG AAAAACCGTC TATCAGGGCT ATGGCCCACT 3540

ACGTGAACCA TCACCCTAAT CAAGTTTTTT GGGGTCGAGG TGCCGTAAAG CACTAAATCG 3600

GAACCCTAAA GGGAGCCCCC GATTTAGAGC TTGACGGGGA AAGCCGGCGA ACGTGGCGAG 3660

AAAGGAAGGG AAGAAAGCGA AAGGAGCGGG CGCTAGGGCG CTGGCAAGTG TAGCGGTCAC 3720

GCTGCGCGTA ACCACCACAC CCGCCGCGCT TAATGCGCCG CTACAGGGCG CGTCCGGATC 3780

CTGCCTCGCG CGTTTCGGTG ATGACGGTGA AAACCTCTGA CACATGCAGC TCCCGGAGAC 3840

GGTCACAGCT TGTCTGTAAG CGGATGCCGG GAGCAGACAA GCCCGTCAGG GCGCGTCAGC 3900

GGGTGTTGGC GGGTGTCGGG GCGCAGCCAT GACCCAGTCA CGTAGCGATA GCGGAGTGTA 3960

TACTGGCTTA ACTATGCGGC ATCAGAGCAG ATTGTACTGA GAGTGCACCA TATGCGGTGT 4020

GAAATACCGC ACAGATGCGT AAGGAGAAAA TACCGCATCA GGCGCTCTTC CGCTTCCTCG 4080

CTCACTGACT CGCTGCGCTC GGTCGTTCGG CTGCGGCGAG CGGTATCAGC TCACTCAAAG 4140

GCGGTAATAC GGTTATCCAC AGAATCAGGG GATAACGCAG GAAAGAACAT GTGAGCAAAA 4200

GGCCAGCAAA AGGCCAGGAA CCGTAAAAAG GCCGCGTTGC TGGCGTTTTT CCATAGGCTC 4260

CGCCCCCCTG ACGAGCATCA CAAAAATCGA CGCTCAAGTC AGAGGTGGCG AAACCCGACA 4320

GGACTATAAA GATACCAGGC GTTTCCCCCT GGAAGCTCCC TCGTGCGCTC TCCTGTTCCG 4380

ACCCTGCCGC TTACCGGATA CCTGTCCGCC TTTCTCCCTT CGGGAAGCGT GGCGCTTTCT 4440

CATAGCTCAC GCTGTAGGTA TCTCAGTTCG GTGTAGGTCG TTCGCTCCAA GCTGGGCTGT 4500

GTGCACGAAC CCCCCGTTCA GCCCGACCGC TGCGCCTTAT CCGGTAACTA TCGTCTTGAG 4560

TCCAACCCGG TAAGACACGA CTTATCGCCA CTGGCAGCAG CCACTGGTAA CAGGATTAGC 4620

AGAGCGAGGT ATGTAGGCGG TGCTACAGAG TTCTTGAAGT GGTGGCCTAA CTACGGCTAC 4680

ACTAGAAGGA CAGTATTTGG TATCTGCGCT CTGCTGAAGC CAGTTACCTT CGGAAAAAGA 4740

GTTGGTAGCT CTTGATCCGG CAAACAAACC ACCGCTGGTA GCGGTGGTTT TTTTGTTTGC 4800

AAGCAGCAGA TTACGCGCAG AAAAAAAGGA TCTCAAGAAG ATCCTTTGAT CTTTTCTACG 4860

GGGTCTGACG CTCAGTGGAA CGAAAACTCA CGTTAAGGGA TTTTGGTCAT GAGATTATCA 4920

AAAAGGATCT TCACCTAGAT CCTTTTAAAT TAAAAATGAA GTTTTAAATC AATCTAAAGT 4980

ATATATGAGT AAACTTGGTC TGACAGTTAC CAATGCTTAA TCAGTGAGGC ACCTATCTCA 5040

GCGATCTGTC TATTTCGTTC ATCCATAGTT GCCTGACTCC CCGTCGTGTA GATAACTACG 5100

ATACGGGAGG GCTTACCATC TGGCCCCAGT GCTGCAATGA TACCGCGAGA CCCACGCTCA 5160

CCGGCTCCAG ATTTATCAGC AATAAACCAG CCAGCCGGAA GGGCCGAGCG CAGAAGTGGT 5220

CCTGCAACTT TATCCGCCTC CATCCAGTCT ATTAATTGTT GCCGGGAAGC TAGAGTAAGT 5280

AGTTCGCCAG TTAATAGTTT GCGCAACGTT GTTGCCATTG CTGCAGGCAT CGTGGTGTCA 5340

CGCTCGTCGT TTGGTATGGC TTCATTCAGC TCCGGTTCCC AACGATCAAG GCGAGTTACA 5400

TGATCCCCCA TGTTGTGCAA AAAAGCGGTT AGCTCCTTCG GTCCTCCGAT CGTTGTCAGA 5460

AGTAAGTTGG CCGCAGTGTT ATCACTCATG GTTATGGCAG CACTGCATAA TTCTCTTACT 5520

GTCATGCCAT CCGTAAGATG CTTTTCTGTG ACTGGTGAGT ACTCAACCAA GTCATTCTGA 5580

GAATAGTGTA TGCGGCGACC GAGTTGCTCT TGCCCGGCGT CAACACGGGA TAATACCGCG 5640

CCACATAGCA GAACTTTAAA AGTGCTCATC ATTGGAAAAC GTTCTTCGGG GCGAAAACTC 5700

TCAAGGATCT TACCGCTGTT GAGATCCAGT TCGATGTAAC CCACTCGTGC ACCCAACTGA 5760

TCTTCAGCAT CTTTTACTTT CACCAGCGTT TCTGGGTGAG CAAAAACAGG AAGGCAAAAT 5820

GCCGCAAAAA AGGGAATAAG GGCGACACGG AAATGTTGAA TACTCATACT CTTCCTTTTT 5880

CAATATTATT GAAGCATTTA TCAGGGTTAT TGTCTCATGA GCGGATACAT ATTTGAATGT 5940

ATTTAGAAAA ATAAACAAAT AGGGGTTCCG CGCACATTTC CCCGAAAAGT GCCACCTGAC 6000

GTCTAAGAAA CCATTATTAT CATGACATTA ACCTATAAAA ATAGGCGTAT CACGAGGCCC 6060

TTTCGTCTTC AA 6072

CLAIMS:

1. A humanized antibody, wherein the complementary determining regions (CDRs) of a non-human antibody are grafted onto a human framework comprising the $V_L\kappa$ subgroup I ($V_L\kappa$I) and V_H subgroup III (V_H III), wherein of the V_L domain, at least one of representatively numbered residues 4 and 71 are substituted with an amino acid which differs from the amino acid at that position, and of the V_H domain, at least three of representatively numbered residues 24,37,67,69,71,73,75, 76, 78, 93 and 94 are substituted with an amino acid which differs from the amino acid at that position.

2. The humanized antibody of claim 1, wherein the antibody is to vascular endothelial growth factor.

3. The humanized antibody of claim 2, wherein the V_L domain has the sequence set forth in SEQ ID NO: 8 and the V_H domain has the sequence set forth in SEQ ID NO: 11.

4. The humanized antibody of Claim 3, wherein residue 46, leucine, of SEQ ID NO: 8 is substituted by valine.

5. The humanized antibody of Claim 3, wherein in SEQ ID NO: 8, residue 4, methionine, is substituted by leucine and residue 71, tyrosine, is substituted with phenylalanine; and in SEQ ID NO: 11, residue 67, phenylalanine, is substituted by threonine.

6. The humanized antibody of claim 2, wherein the V_L domain has the sequence set forth in SEQ ID NO: 7 and the V_H domain has the sequence set forth in SEQ ID NO: 10, wherein in SEQ ID NO: 7, residue 71, phenylalanine, is substituted by tyrosine, and in SEQ ID NO: 10, residue 37, valine, is substituted by isoleucine, residue 78, leucine, is substituted by valine, and residue 94, arginine, is substituted by lysine.

7. The humanized antibody of claim 6, wherein in SEQ ID NO: 7, residue 4, methionine, is substituted by leucine.

8. The humanized antibody of claim 7, wherein in SEQ ID NO: 7, residue 71, tyrosine, is substituted by phenylalanine and wherein in SEQ ID NO: 10, residue 67, phenylalanine, is substituted by threonine.

9. A method of humanizing a non-human antibody comprising the steps of:

grafting complementary determining regions (CDRs) of a non-human antibody onto a human framework comprising the $V_L\kappa$ subgroup I ($V_L\kappa$I) and V_H subgroup III (V_H III);

substituting in the V_L domain, at least one of residues 4 and 71 by an amino acid that is different from the amino acid at that position;

substituting in the V_H domain, at least three of residues 24,37,67,69,71,73,75, 76, 78, 93 and 94 by an amino acid that is different from the amino acid at that position.

10. The method of claim 9, wherein the antibody is for vascular endothelial growth factor.

11. The method of claim 10, wherein the V_L domain has the sequence set forth in SEQ ID NO: 8 and the V_H domain has the sequence set forth in SEQ ID NO: 11.

12. The method of claim 11, wherein residue 46, leucine, of SEQ ID NO: 8 is substituted by valine.

13. The method of claim 11, wherein in SEQ ID NO: 8, residue 4, methionine, is substituted by leucine and residue 71, tyrosine, is substituted by phenylalanine; and in SEQ ID NO: 11, residue 67, phenylalanine, is substituted by threonine.

14. The method of claim 10, wherein the V_L domain has the sequence set forth in SEQ ID NO: 7 and the V_H domain has the sequence set forth in SEQ ID NO: 10, wherein in SEQ ID NO: 7, residue 71, phenylalanine, is substituted by tyrosine, and in SEQ ID NO: 10, residue 37, valine, is substituted by isoleucine, residue 78, leucine, is substituted by valine, and residue 94, arginine, is substituted by lysine.

15. The method of claim 14, wherein in SEQ ID NO: 7, residue 4, methionine, is substituted by leucine.

16. The method of claim 15, wherein in SEQ ID NO: 7, residue 71, tyrosine, is substituted by phenylalanine.

17. The method of claim 10 further comprising the steps of:

displaying the V_L and V_H domains by substitutions on a phagemid;

determining whether VEGF will to the bind to the V_L and V_H domains by substitutions;

selecting humanized antibodies which will bind to VEGF.

18. A method for inhibiting tumor growth by inhibiting mitogenic signaling comprising administering the humanized antibody of claim 1 to a tumor.

19. The humanized antibody of Claim 1 wherein the antibody is encoded by a nucleic acid molecule which hybridizes under high stringency conditions to a nucleic acid molecule having the sequence set forth in SEQ ID NO: 14.

20. The humanized antibody of Claim 1 encoded by a nucleic acid molecule having the sequence set forth in SEQ ID NO: 14.

V_L domain

```
                10        20        30        40
A4.6.1   DIQMTQTTSSLSASLGDRVIISCSASQDISNYLNWYQQKP
               **       *    * *
hu2.0    DIQMTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQKP

hu2.10   DIQMTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQKP


                50        60        70        80
A4.6.1   DGTVKVLIYFTSSLHSGVPSRFSGSGSGTDYSLTISNLEP
         **** *                          **    * *
hu2.0    GKAPKLLIYFTSSLHSGVPSRFSGSGSGTDFTLTISSLQP
                                          •
hu2.10   GKAPKLLIYFTSSLHSGVPSRFSGSGSGTDYTLTISSLQP


                90       100
A4.6.1   EDIATYYCQQYSTVPWTFGGGTKLEIK
           *                *   *
hu2.0    EDFATYYCQQYSTVPWTFGQGTKVEIK

hu2.10   EDFATYYCQQYSTVPWTFGQGTKVEIK
```

V_H domain

```
                10        20        30        40
A4.6.1   EIQLVQSGPELKQPGETVRISCKASGYTFTNYGMNWVKQA
          *   *  ** *   *** *   *                *
hu2.0    EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWVRQA
                                             •
hu2.10   EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWIRQA


                50 a      60        70        80
A4.6.1   PGKGLKWMGWINTYTGEPTYAADFKRRFTFSLETSASTAYL
              * *                  * *** ** *
hu2.0    PGKGLEWVGWINTYTGEPTYAADFKRRFTISRDNSKNTLYL
                                     • • •• •
hu2.10   PGKGLEWVGWINTYTGEPTYAADFKRRFTISLDTSASTVYL


           abc      90      100abcdef       110
A4.6.1   QISNLKNDDTATYFCAKYPHYYGSSHWYFDVWGAGTTVTVSS
          *** ***   * *  *                * *
hu2.0    QMNSLRAEDTAVYYCARYPHYYGSSHWYFDVWGQGTLVTVSS
                       •
hu2.10   QMNSLRAEDTAVYYCAKYPHYYGSSHWYFDVWGQGTLVTVSS
```

FIG._1

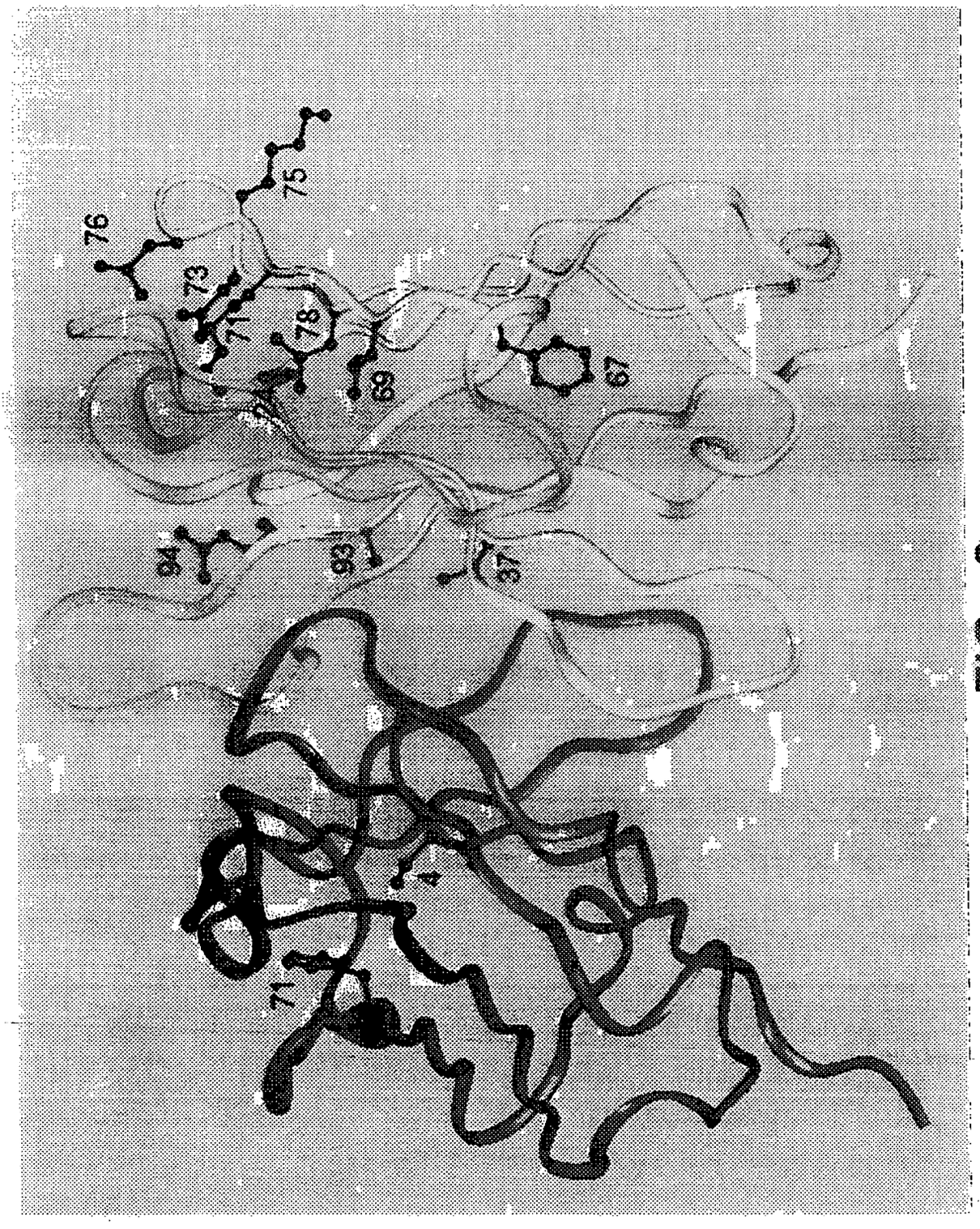

FIG._2

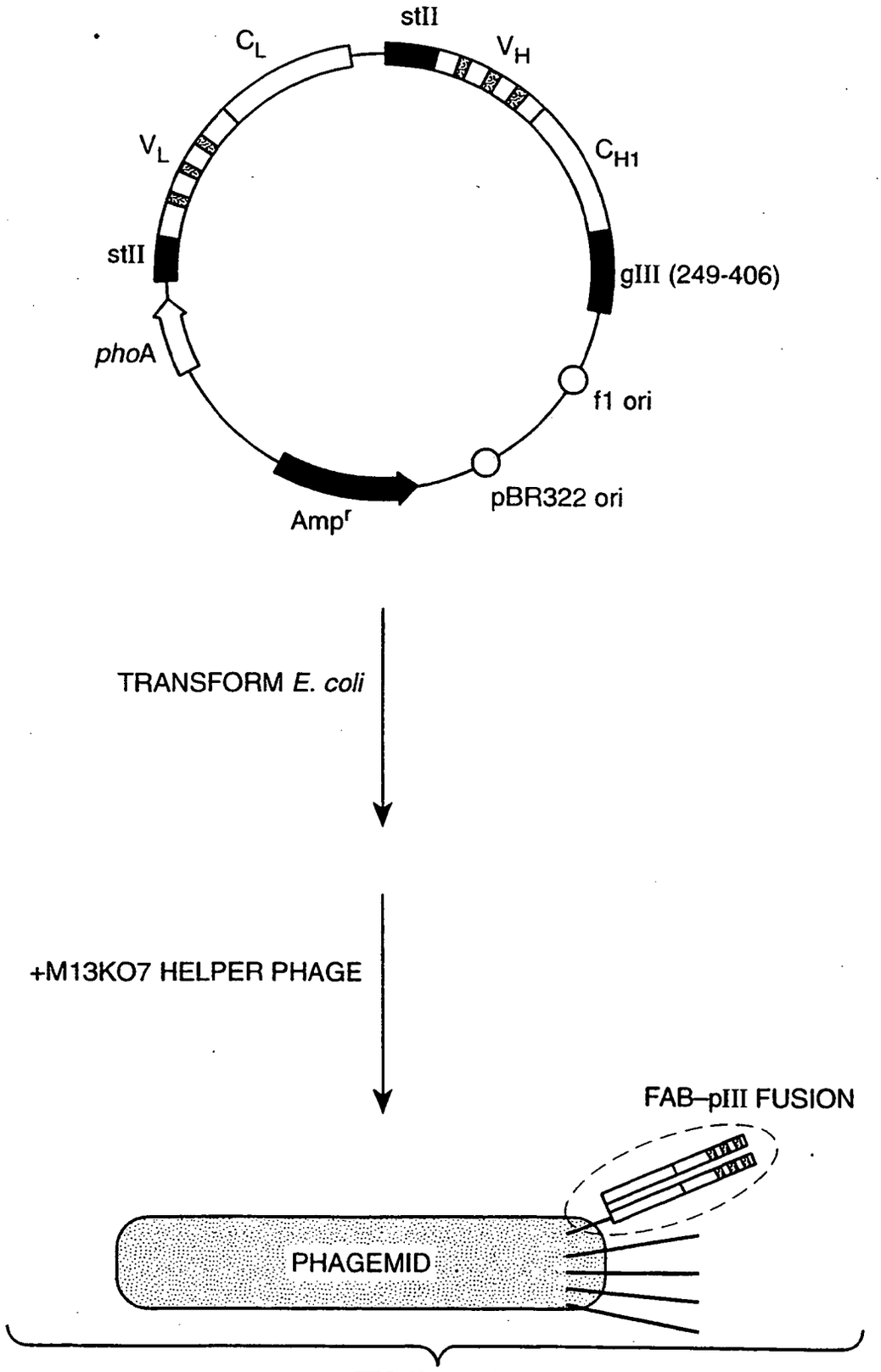

FIG._3

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(30) Priority Data:
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(71) Applicant *(for all designated States except US)*: GENENTECH, INC. [US/US]; One DNA Way, South San Francisco, CA 94080 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: WELLS, James, A. [US/US]; 1341 Columbus Avenue, Burlingame, CA 94010 (US). BACA, Manuel [AU/US]; Apartment #H3, 888 Foster City Boulevard, Foster City, CA 94404 (US). PRESTA, Leonard, G. [US/US]; Apartment 206, 1900 Gough, San Francisco, CA 94109 (US).

(74) Agents: DREGER, Walter, H. et al.; Flehr, Hohbach, Test, Albritton & Herbert LLP, Suite 3400, 4 Embarcadero Center, San Francisco, CA 94111-4187 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
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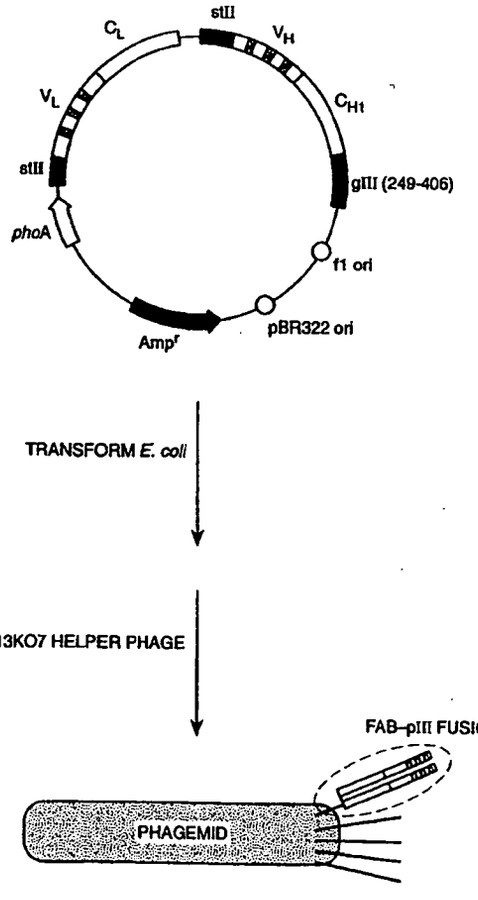
(54) Title: HUMANIZED ANTIBODIES AND METHODS FOR FORMING HUMANIZED ANTIBODIES

(57) Abstract

Described herein is a humanized antibody to vascular endothelial growth factor (VEGF). Also described herein is a method for rapidly producing and identifying framework mutations which improve the binding of humanized antibodies to their cognate antigens. In a preferred embodiment, non-human CDRs are grafted onto a human $V_I\kappa I$-$V_H III$ framework. Random mutagenesis of a small set of critical framework residues is also performed followed by monovalent display of the resultant library of antibody molecules on the surface of filamentous phage. The optimal framework sequences are then identified by affinity-based selection. Optionally, the selected antibodies can be further mutated so as to replace vernier residues which sit at the V_L-V_H interface by residues which match the non-human parent antibody. The methods described herein can be applied to any non-human antibody. Accordingly, humanized antibodies are provided.



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INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/US 98/06724

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K16/22 C12N15/13 C12N15/63 C12N15/70 A61K39/395

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	WO 92 22653 A (GENENTECH INC) 23 December 1992 the whole document and specially: see SEQ.ID.N. 17 and 18 see page 5, line 24 - page 7, line 35 see page 9, line 22 - page 10, line 4; figure 5	3,11 1,2,9,10
Y	KIM ET AL.,: "Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumor growth in vivo" NATURE, vol. 362, 1993, page 841 XP002013864 London, GB cited in the application see abstract	1,2,9,10

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
18 September 1998	02/10/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Mateo Rosell, A.M.

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INTERNATIONAL SEARCH REPORT

Inte onal Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication.where appropriate, of the relevant passages	Relevant to claim No.
A	US 5.580 723 A (WELLS JAMES A ET AL) . 3 December 1996 see figures 12A-J	19,20
A	WO 94 04679 A (GENENTECH INC) 3 March 1994 see page 1-73	1,9
A	GB 2 268 744 A (CELLTECH LTD) 19 January 1994 see abstract see page 4, paragraph 3 - page 6, paragraph 1	1,9
A	M.M. BENDIG: "Humanization of rodent monoclonal antibodies" METHODS: A COMPANION TO METHODS IN ENZYMOLOGY, vol. 8, 1995, pages 83-93, XP000647344 New York, NY, US see the whole document	1,10
P,X	M. BACA ET AL., : "Antibody humanization using monovalent phage display" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 272, no. 16, 18 April 1997, pages 10678-10684, XP002077471 see the whole document	1-14

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9222653 A	23-12-1992	AU 675916 B	27-02-1997
		AU 2250992 A	12-01-1993
		CA 2103059 A	15-12-1992
		EP 0590058 A	06-04-1994
		JP 6508267 T	22-09-1994
		WO 9404679 A	03-03-1994
US 5580723 A	03-12-1996	US 5766854 A	16-06-1998
		US 5534617 A	09-07-1996
		EP 0397834 A	22-11-1990
		JP 4502454 T	07-05-1992
		WO 9004788 A	03-05-1990
		CA 2001774 A	28-04-1990
		US 5688666 A	18-11-1997
WO 9404679 A	03-03-1994	AU 675916 B	27-02-1997
		AU 2250992 A	12-01-1993
		EP 0590058 A	06-04-1994
		JP 6508267 T	22-09-1994
		CA 2103059 A	15-12-1992
		WO 9222653 A	23-12-1992
		AU 5083193 A	15-03-1994
GB 2268744 A	19-01-1994	AT 129017 T	15-10-1995
		AT 124459 T	15-07-1995
		AT 159299 T	15-11-1997
		AU 664801 B	30-11-1995
		AU 6461294 A	22-12-1994
		AU 646009 B	03-02-1994
		AU 6974091 A	24-07-1991
		AU 649645 B	02-06-1994
		AU 7033091 A	24-07-1991
		AU 631481 B	26-11-1992
		AU 7048691 A	24-07-1991
		BG 60462 B	28-04-1995
		CA 2037607 A	07-09-1992
		CA 2046904 A	22-06-1991
		CA 2050479 A,C	22-06-1991
		DE 69020544 D	03-08-1995
		DE 69020544 T	18-01-1996

Information on patent family members

International Application No
PCT/US 98/06724

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
GB 2268744 A		DE 69022982 D	16-11-1995
		DE 69022982 T	28-03-1996
		DE 69031591 D	20-11-1997
		DE 69031591 T	12-03-1998
		DK 460167 T	20-11-1995
		DK 460171 T	28-08-1995
		DK 460178 T	22-12-1997
		EP 0460167 A	11-12-1991
		EP 0460171 A	11-12-1991
		EP 0460178 A	11-12-1991
		EP 0620276 A	19-10-1994
		EP 0626390 A	30-11-1994
		ES 2079638 T	16-01-1996
		ES 2074701 T	16-09-1995
		ES 2112270 T	01-04-1998
		WO 9109966 A	11-07-1991
		WO 9109967 A	11-07-1991
		WO 9109968 A	11-07-1991
		GB 2246781 A,B	12-02-1992
		GB 2246570 A,B	05-02-1992
		GB 2268745 A,B	19-01-1994
		GR 3017734 T	31-01-1996
		GR 3025781 T	31-03-1998
		JP 4505398 T	24-09-1992
		JP 4506458 T	12-11-1992
		JP 5500312 T	28-01-1993

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(30) Priority Data:

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08/908,469	6 August 1997 (06.08.97)	US

(71) Applicant *(for all designated States except US)*: GENENTECH, INC. [US/US]; One DNA Way, South San Francisco, CA 94080 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: BACA, Manuel [AU/US]; Apartment #H3, 888 Foster City Boulevard, Foster City, CA 94404 (US). WELLS, James, A. [US/US]; 1341 Columbus Avenue, Burlingame, CA 94010 (US). PRESTA, Leonard, G. [US/US]; Apartment 206, 1900 Gough Street, San Francisco, CA 94109 (US). LOWMAN, Henry, B. [US/US]; 400 San Juan Avenue, El Granada, CA 94018 (US). CHEN, Yvonne, Man-Yee [CA/US]; 1951 O'Farrell Street #321, San Mateo, CA 94403 (US).

(74) Agents: DREGER, Walter, H. et al.; Flehr, Hohbach, Test, Albritton & Herbert LLP, Suite 400, 4 Embarcadero Center, San Francisco, CA 94111-4187 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

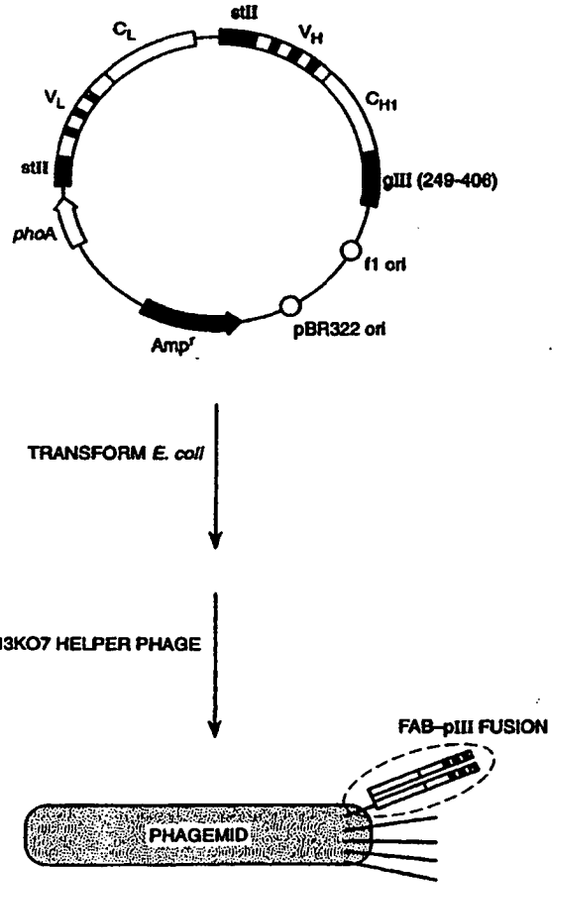
Published

Without international search report and to be republished upon receipt of that report.

(54) Title: ANTI-VEGF ANTIBODIES

(57) Abstract

Humanized and variant anti-VEGF antibodies and various uses therefor are disclosed. The anti-VEGF antibodies have strong binding affinities for VEGF; inhibit VEGF-induced proliferation of endothelial cells *in vitro*, and inhibit tumor growth *in vivo*.



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ANTI-VEGF ANTIBODIES

Cross References

This application is a continuation-in-part of co-pending U.S. Application No. 08/833,504, filed April 7, 1997, which application is incorporated herein by reference and to which application priority is claimed under 35 U.S.C. §120.

BACKGROUND OF THE INVENTION

Field of the Invention

This invention relates generally to anti-VEGF antibodies and, in particular, to humanized anti-VEGF antibodies and variant anti-VEGF antibodies.

Description of Related Art

It is now well established that angiogenesis is implicated in the pathogenesis of a variety of disorders. These include solid tumors, intraocular neovascular syndromes such as proliferative retinopathies or age-related macular degeneration (AMD), rheumatoid arthritis, and psoriasis (Folkman *et al. J. Biol. Chem.* 267:10931-10934 (1992); Klagsbrun *et al. Annu. Rev. Physiol.* 53:217-239 (1991); and Garner A, *Vascular diseases. In: Pathobiology of ocular disease. A dynamic approach.* Garner A, Klintworth GK, Eds. 2nd Edition Marcel Dekker, NY, pp 1625-1710 (1994)). In the case of solid tumors, the neovascularization allows the tumor cells to acquire a growth advantage and proliferative autonomy compared to the normal cells. Accordingly, a correlation has been observed between density of microvessels in tumor sections and patient survival in breast cancer as well as in several other tumors (Weidner *et al. N Engl J Med* 324:1-6 (1991); Horak *et al. Lancet* 340:1120-1124 (1992); and Macchiarini *et al. Lancet* 340:145-146 (1992)).

The search for positive regulators of angiogenesis has yielded many candidates, including aFGF, bFGF, TGF-α, TGF-β, HGF, TNF-α, angiogenin, IL-8, etc. (Folkman *et al.* and Klagsbrun *et al*). The negative regulators so far identified include thrombospondin (Good *et al. Proc. Natl. Acad. Sci. USA.* 87:6624-6628 (1990)), the 16-kilodalton N-terminal fragment of prolactin (Clapp *et al. Endocrinology*, 133:1292-1299 (1993)), angiostatin (O'Reilly *et al. Cell*, 79:315-328 (1994)) and endostatin (O'Reilly *et al. Cell*, 88:277-285 (1996)).

Work done over the last several years has established the key role of vascular endothelial growth factor (VEGF) in the regulation of normal and abnormal angiogenesis (Ferrara *et al. Endocr. Rev.* 18:4-25 (1997)). The finding that the loss of even a single VEGF allele results in embryonic lethality points to an irreplaceable role played by this factor in the development and differentiation of the vascular system (Ferrara *et al.*). Furthermore, VEGF has been shown to be a key mediator of neovascularization associated with tumors and intraocular disorders (Ferrara *et al.*). The VEGF mRNA is overexpressed by the majority of human tumors examined (Berkman *et al. J Clin Invest* 91:153-159 (1993); Brown *et al. Human Pathol.*. 26:86-91 (1995); Brown *et al. Cancer Res.* 53:4727-4735 (1993); Mattern *et al. Brit. J. Cancer*. 73:931-934 (1996); and Dvorak *et al. Am J. Pathol.* 146:1029-1039 (1995)). Also, the concentration of VEGF in eye fluids are highly correlated to the presence of active proliferation of blood vessels in patients with diabetic and other ischemia-related retinopathies (Aiello *et al. N. Engl. J. Med.* 331:1480-1487 (1994)). Furthermore, recent studies have demonstrated the localization of VEGF in choroidal neovascular membranes in patients affected by AMD (Lopez *et al. Invest. Ophtalmo. Vis. Sci.* 37:855-868 (1996)). Anti-VEGF neutralizing antibodies suppress the growth of a variety of human tumor cell lines in nude mice (Kim *et al. Nature* 362:841-844 (1993); Warren *et al. J. Clin. Invest.* 95:1789-1797 (1995); Borgström *et al. Cancer Res.* 56:4032-4039 (1996); and Melnyk *et al. Cancer Res.* 56:921-924 (1996)) and also inhibit intraocular angiogenesis in models of ischemic retinal disorders (Adamis *et al. Arch. Ophthalmol.* 114:66-71 (1996)). Therefore, anti-VEGF monoclonal antibodies or other inhibitors of VEGF action are promising candidates for the treatment of solid tumors and various intraocular neovascular disorders.

SUMMARY OF THE INVENTION

This application describes humanized anti-VEGF antibodies and anti-VEGF antibody variants with desirable properties from a therapeutic perspective, including strong binding affinity for VEGF; the ability to inhibit VEGF-induced proliferation of endothelial cells *in vitro*; and the ability to inhibit VEGF-induced angiogenesis *in vivo*.

The preferred humanized anti-VEGF antibody or variant anti-VEGF antibody herein binds human VEGF with a K_d value of no more than about 1×10^{-8}M and preferably no more than about 5×10^{-9}M. In addition, the humanized or variant anti-VEGF antibody may have an ED50 value of no more than about 5nM for inhibiting VEGF-induced proliferation of

endothelial cells *in vitro*. The humanized or variant anti-VEGF antibodies of particular interest herein are those which inhibit at least about 50% of tumor growth in an A673 *in vivo* tumor model, at an antibody dose of 5mg/kg.

In one embodiment, the anti-VEGF antibody has a heavy and light chain variable domain, wherein the heavy chain variable domain comprises hypervariable regions with the following amino acid sequences: CDRH1 (GYX_1FTX_2YGMN, wherein X_1 is T or D and X_2 is N or H; SEQ ID NO:128), CDRH2 (WINTYTGEPTYAADFKR; SEQ ID NO:2) and CDRH3 ($YPX_1YYGX_2SHWYFDV$, wherein X_1 is Y or H and X_2 is S or T; SEQ ID NO:129). For example, the heavy chain variable domain may comprise the amino acid sequences of CDRH1 (GYTFTNYGMN; SEQ ID NO:1), CDRH2 (WINTYTGEPTYAADFKR; SEQ ID NO:2) and CDRH3 (YPHYYGSSHWYFDV; SEQ ID NO:3). Preferably, the three heavy chain hypervariable regions are provided in a human framework region, *e.g.*, as a contiguous sequence represented by the following formula: FR1-CDRH1-FR2-CDRH2-FR3-CDRH3-FR4.

The invention further provides an anti-VEGF antibody heavy chain variable domain comprising the amino acid sequence:

EVQLVESGGGLVQPGGSLRLSCAASGYX$_1$FTX$_2$YGMNWVRQAPGKGLEWVGWI NTYTGEPTYAADFKRRFTFSLDTSKSTAYLQMNSLRAEDTAVYYCAKYPX$_3$YYG X$_4$SHWYFDVWGQGTLVTVSS (SEQ ID NO:125), wherein X_1 is T or D; X_2 is N or H; X_3 is Y or H and X_4 is S or T. One particularly useful heavy chain variable domain sequence is that of the F(ab)-12 humanized antibody of Example 1 and comprises the heavy chain variable domain sequence of SEQ ID NO:7. Such preferred heavy chain variable domain sequences may be combined with the following preferred light chain variable domain sequences or with other light chain variable domain sequences, provided that the antibody so produced binds human VEGF.

The invention also provides preferred light chain variable domain sequences which may be combined with the above-identified heavy chain variable domain sequences or with other heavy chain variable domain sequences, provided that the antibody so produced retains the ability to bind to human VEGF. For example, the light chain variable domain may comprise hypervariable regions with the following amino acid sequences: CDRL1 (SASQDISNYLN; SEQ ID NO:4), CDRL2 (FTSSLHS; SEQ ID NO:5) and CDRL3 (QQYSTVPWT; SEQ ID NO:6). Preferably, the three light chain hypervariable regions are

provided in a human framework region, *e.g.*, as a contiguous sequence represented by the following formula: FR1-CDRL1-FR2-CDRL2-FR3-CDRL3-FR4.

In one embodiment, the invention provides a humanized anti-VEGF antibody light chain variable domain comprising the amino acid sequence:

DIQX$_1$TQSPSSLSASVGDRVTITCSASQDISNYLNWYQQKPGKAPKVLIYFTSSLHS GVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYSTVPWTFGQGTKVEIKR (SEQ ID NO:124), wherein X_1 is M or L. One particularly useful light chain variable domain sequence is that of the F(ab)-12 humanized antibody of Example 1 and comprises the light chain variable domain sequence of SEQ ID NO:8.

The invention also provides a variant of a parent anti-VEGF antibody (which parent antibody is preferably a humanized or human anti-VEGF antibody), wherein the variant binds human VEGF and comprises an amino acid substitution in a hypervariable region of the heavy or light chain variable domain of the parent anti-VEGF antibody. The variant preferably has one or more substitution(s) in one or more hypervariable region(s) of the anti-VEGF antibody. Preferably, the substitution(s) are in the heavy chain variable domain of the parent antibody. For example, the amino acid subsition(s) may be in the CDRH1 and/or CDRH3 of the heavy chain variable domain. Preferably, there are substitutions in both these hypervariable regions. Such "affinity matured" variants are demonstrated herein to bind human VEGF more strongly than the parent anti-VEGF antibody from which they are generated, *i.e.*, they have a K_d value which is significantly less than that of the parent anti-VEGF antibody. Preferably, the variant has an ED50 value for inhibiting VEGF-induced proliferation of endothelial cells *in vitro* which is at least about 10 fold lower, preferably at least about 20 fold lower, and most preferably at least about 50 fold lower, than that of the parent anti-VEGF antibody. One particularly prefered variant is the Y0317 variant of Example 3, which has a CDRH1 comprising the amino acid sequence:GYDFTHYGMN (SEQ ID NO:126) and a CDRH3 comprising the amino acid sequence:YPYYYGTSHWYFDV (SEQ ID NO:127). These hypervariable regions and CDRH2 are generally provided in a human framework region, *e.g.*, resulting in a heavy chain variable domain comprising the amino acid sequence of SEQ ID NO:116. Such heavy chain variable domain sequences are optionally combined with a light chain variable domain comprising the amino acid sequence of SEQ ID NO:124, and preferably the light chain variable domain amino acid sequence of SEQ ID NO:115.

Various forms of the antibody are contemplated herein. For example, the anti-VEGF antibody may be a full length antibody (*e.g.* having an intact human Fc region) or an antibody fragment (*e.g.* a Fab, Fab' or $F(ab')_2$). Furthermore, the antibody may be labeled with a detectable label, immobilized on a solid phase and/or conjugated with a heterologous compound (such as a cytotoxic agent).

Diagnostic and therapeutic uses for the antibody are contemplated. In one diagnostic application, the invention provides a method for determining the presence of VEGF protein comprising exposing a sample suspected of containing the VEGF protein to the anti-VEGF antibody and determining binding of the antibody to the sample. For this use, the invention provides a kit comprising the antibody and instructions for using the antibody to detect the VEGF protein.

The invention further provides: isolated nucleic acid encoding the antibody; a vector comprising that nucleic acid, optionally operably linked to control sequences recognized by a host cell transformed with the vector; a host cell comprising that vector; a process for producing the antibody comprising culturing the host cell so that the nucleic acid is expressed and, optionally, recovering the antibody from the host cell culture (*e.g.* from the host cell culture medium). The invention also provides a composition comprising the anti-VEGF antibody and a pharmaceutically acceptable carrier or diluent. The composition for therapeutic use is sterile and may be lyophilized. The invention further provides a method for treating a mammal suffering from a tumor or retinal disorder, comprising administering a therapeutically effective amount of the anti-VEGF antibody to the mammal.

BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1A and 1B depict the amino acid sequences of variable heavy domain (SEQ ID NO:9) and light domain (SEQ ID NO:10) of muMAbVEGF A.4.6.1, variable heavy domain (SEQ ID NO:7) and light domain (SEQ ID NO:8) of humanized F(ab) (F(ab)-12) and human consensus frameworks (hum III for heavy subgroup III (SEQ ID NO:11); humκ1 for light κ subgroup I (SEQ ID NO:12)). Fig. 1A aligns variable heavy domain sequences and Fig. 1B aligns variable light domain sequences. Asterisks indicate differences between humanized F(ab)-12 and the murine MAb or between F(ab)-12 and the human framework. Complementarity Determining Regions (CDRs) are underlined.

Fig. 2 is a ribbon diagram of the model of humanized F(ab)-12 VL and VH domains. VL domain is shown in brown with CDRs in tan. The sidechain of residue L46 is shown in yellow. VH domain is shown in purple with CDRs in pink. Sidechains of VH residues changed from human to murine are shown in yellow.

Fig. 3 depicts inhibition of VEGF-induced mitogenesis by humanized anti-VEGF F(ab)-12 from Example 1. Bovine adrenal cortex-derived capillary endothelial cells were seeded at the density of 6 X 10^3 cells/well in six well plates, as described in Example 1. Either muMAb VEGF A.4.6.1 or rhuMAb VEGF (IgG1; F(ab)-12) was added at the indicated concentrations. After 2-3 hours, rhVEGF165 was added at the final concentration of 3 ng/ml. After five or six days, cells were trypsinized and counted. Values shown are means of duplicate determinations. The variation from the mean did not exceed 10%.

Fig. 4 shows inhibition of tumor growth *in vivo* by humanized anti-VEGF F(ab)-12 from Example 1. A673 rhabdomyosarcoma cells were injected in BALB/c nude mice at the density of 2 x 10^6 per mouse. Starting 24 hours after tumor cell inoculation, animals were injected with a control MAb, muMAb VEGF A4.6.1 or rhuVEGF MAb (IgG1; F(ab)-12) twice weekly, intra peritoneally. The dose of the control Mab was 5 mg/kg; the anti-VEGF MAbs were given at 0.5 or 5 mg/kg, as indicated (n = 10). Four weeks after tumor cell injection, animals were euthanized and tumors were removed and weighed. *: significant difference when compared to the control group by ANOVA ($p < 0.05$).

Figs. 5A and 5B show the acid sequences of the light and heavy variable domains respectively of murine antibody A4.6.1 (SEQ ID NO:10 for the VL and SEQ ID NO:9 for the VH) and humanized A4.6.1 variants hu2.0 (SEQ ID NO:13 for the VL and SEQ ID NO:14 for the VH) and hu2.10 (SEQ ID NO:15 for the VL and SEQ ID NO:16 for the VH) from Example 2. Sequence numbering is according to Kabat *et al., Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991) and mismatches are indicated by asterisks (murine A4.6.1 vs hu2.0) or bullets (hu2.0 vs hu2.10). Variant hu2.0 contains only the CDR sequences (bold) from the murine antibody grafted onto a human light chain κ subgroup I consensus framework (SEQ ID NO:12) and heavy chain subgroup III consensus framework (SEQ ID NO:11). hu2.10 was the consensus humanized clone obtained from phage sorting experiments described herein.

Fig. 6 depicts framework residues targeted for randomization in Example 2.

Fig. 7 depicts the phagemid construct for surface display of Fab-pIII fusions on phage. The phagemid encodes a humanized version of the Fab fragment for antibody A4.6.1 fused to a portion of the M13 gene III coat protein. The fusion protein consists of the Fab joined at the carboxyl terminus of the heavy chain to a single glutamine residue (from suppression of an amber codon in *supE E. coli)*, then the C-terminal region of the gene III protein (residues 249-406). Transformation into F^+ *E. coli*, followed by superinfection with M13KO7 helper phage, produces phagemid particles in which a small proportion of these display a single copy of the fusion protein.

Figs. 8A-E depict the double stranded nucleotide sequence (SEQ ID NO:99) for phage-display antibody vector phMB4-19-1.6 in Example 3 and the amino acid sequence encoded thereby (SEQ ID NO:100).

Figs. 9A and 9B depict an alignment of the amino acid sequences for the light and heavy variable domains respectively of affinity matured anti-VEGF variants in Example 3, compared to F(ab)-12 of Example 1 (SEQ ID NO's 8 and 7 for light and heavy variable domains, respectively). CDRs are underlined and designated by L, light, or H, heavy chain, and numbers 1-3. Residues are numbered sequentially in the VL and VH domains, as opposed to the Kabat numbering scheme. The template molecule, MB1.6 (SEQ ID NO's 101 and 102 for light and heavy variable domains, respectively) is shown, along with variants: H2305.6 (SEQ ID NO's 103 and 104 for light and heavy variable domains, respectively), Y0101 (SEQ ID NO's 105 and 106 for light and heavy variable domains, respectively), and Y0192 (SEQ ID NO's 107 and 108 for light and heavy variable domains, respectively). Differences from F(ab)-12 are shown in shaded boxes.

Figs. 10A and 10B depict an alignment of the amino acid sequences for the light and heavy variable domains respectively of affinity matured anti-VEGF variants from Example 3 compared to F(ab)-12 of Example 1 (SEQ ID NO's 8 and 7 for light and heavy variable domains, respectively). CDRs are underlined and designated by L, light, or H, heavy chain, and numbers 1-3. The variants are designated Y0243-1 (SEQ ID NO's 109 and 110 for light and heavy variable domains, respectively), Y0238-3 (SEQ ID NO's 111 and 112 for light and heavy variable domains, respectively), Y0313-1 (SEQ ID NO's 113 and 114 for light and heavy variable domains, respectively), and Y0317 (SEQ ID NO's 115 and 116 for light and heavy variable domains, respectively). Differences from F(ab)-12 are shown in shaded boxes.

Fig. 11 depicts the results of the HuVEC activity assay in Example 3 for variants Y0238-3, Y0192 and Y0313-1 as well as full length F(ab)-12 from Example 1.

Fig. 12 depicts inhibition of VEGF-induced mitogenesis by full length F(ab)-12 from Example 1 (rhuMAb VEGF), a Fab fragment of F(ab)-12 from Example 1 (rhuFab VEGF), and a Fab fragment of affinity matured variant Y0317 from Example 3 (rhuFab VEGF (affinity matured)).

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

I. Definitions

The term "human VEGF" as used herein refers to the 165-amino acid human vascular endothelial cell growth factor, and related 121-, 189-, and 206-amino acid vascular endothelial cell growth factors, as described by Leung *et al.*, *Science* 246:1306 (1989), and Houck *et al.*, *Mol. Endocrin.* 5:1806 (1991) together with the naturally occurring allelic and processed forms of those growth factors.

The present invention provides anti-VEGF antagonistic antibodies which are capable of inhibiting one or more of the biological activities of VEGF, for example, its mitogenic or angiogenic activity. Antagonists of VEGF act by interfering with the binding of VEGF to a cellular receptor, by incapacitating or killing cells which have been activated by VEGF, or by interfering with vascular endothelial cell activation after VEGF binding to a cellular receptor. All such points of intervention by a VEGF antagonist shall be considered equivalent for purposes of this invention.

The term "VEGF receptor" or "VEGFr" as used herein refers to a cellular receptor for VEGF, ordinarily a cell-surface receptor found on vascular endothelial cells, as well as variants thereof which retain the ability to bind hVEGF. One example of a VEGF receptor is the *fms*-like tyrosine kinase (*flt*), a transmembrane receptor in the tyrosine kinase family. DeVries *et al.*, *Science* 255:989 (1992); Shibuya *et al.*, *Oncogene* 5:519 (1990). The *flt* receptor comprises an extracellular domain, a transmembrane domain, and an intracellular domain with tyrosine kinase activity. The extracellular domain is involved in the binding of VEGF, whereas the intracellular domain is involved in signal transduction. Another example of a VEGF receptor is the *flk-1* receptor (also referred to as KDR). Matthews *et al.*, *Proc. Nat. Acad. Sci.* 88:9026 (1991); Terman *et al.*, *Oncogene* 6:1677 (1991); Terman *et al.*, *Biochem. Biophys. Res. Commun.* 187:1579 (1992). Binding of VEGF to the *flt* receptor

results in the formation of at least two high molecular weight complexes, having apparent molecular weight of 205,000 and 300,000 Daltons. The 300,000 Dalton complex is believed to be a dimer comprising two receptor molecules bound to a single molecule of VEGF.

The term "epitope A4.6.1" when used herein, unless indicated otherwise, refers to the region of human VEGF to which the A4.6.1 antibody disclosed in Kim *et al.*, *Growth Factors* 7:53 (1992) and Kim *et al. Nature* 362:841 (1993), binds.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented.

"Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, *etc.* Preferably, the mammal is human.

"Antibodies" (Abs) and "immunoglobulins" (Igs) are glycoproteins having the same structural characteristics. While antibodies exhibit binding specificity to a specific antigen, immunoglobulins include both antibodies and other antibody-like molecules which lack antigen specificity. Polypeptides of the latter kind are, for example, produced at low levels by the lymph system and at increased levels by myelomas.

"Native antibodies" and "native immunoglobulins" are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies among the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. Each light chain has a variable domain at one end (V_L) and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light- chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light- and heavy-chain variable domains.

The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three

segments called hypervariable regions both in the light chain and the heavy chain variable domains. The more highly conserved portions of variable domains are called the framework region (FR). The variable domains of native heavy and light chains each comprise four FRs (FR1, FR2, FR3 and FR4, respectively), largely adopting a β-sheet configuration, connected by three hypervariable regions, which form loops connecting, and in some cases forming part of, the β-sheet structure. The hypervariable regions in each chain are held together in close proximity by the FRs and, with the hypervariable regions from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat *et al., Sequences of Proteins of Immunological Interest,* 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991), pages 647-669). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody-dependent cellular toxicity.

The term "hypervariable region" when used herein refers to the amino acid residues of an antibody which are responsible for antigen-binding. The hypervariable region comprises amino acid residues from a "complementarity determining region" or "CDR" (*i.e.* residues 24-34 (L1), 50-56 (L2) and 89-97 (L3) in the light chain variable domain and 31-35 (H1), 50-65 (H2) and 95-102 (H3) in the heavy chain variable domain; Kabat *et al., Sequences of Proteins of Immunological Interest,* 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991)) and/or those residues from a "hypervariable loop" (*i.e.* residues 26-32 (L1), 50-52 (L2) and 91-96 (L3) in the light chain variable domain and 26-32 (H1), 53-55 (H2) and 96-101 (H3) in the heavy chain variable domain; Chothia and Lesk *J. Mol. Biol.* 196:901-917 (1987)). "Framework" or "FR" residues are those variable domain residues other than the hypervariable region residues as herein defined.

Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an $F(ab')_2$ fragment that has two antigen-combining sites and is still capable of cross-linking antigen.

"Fv" is the minimum antibody fragment which contains a complete antigen-recognition and -binding site. This region consists of a dimer of one heavy chain and one light chain variable domain in tight, non-covalent association. It is in this configuration that the three hypervariable regions of each variable domain interact to define an antigen-binding site on the surface of the V_H-V_L dimer. Collectively, the six hypervariable regions confer

antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three hypervariable regions specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

The Fab fragment also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxyl terminus of the heavy chain CH1 domain including one or more cysteine(s) from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. $F(ab')_2$ antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

The "light chains" of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (κ) and lambda (λ), based on the amino acid sequences of their constant domains.

Depending on the amino acid sequence of the constant domain of their heavy chains, immunoglobulins can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), *e.g.*, IgG1, IgG2, IgG3, IgG4, IgA, and IgA2. The heavy-chain constant domains that correspond to the different classes of immunoglobulins are called α, δ, ϵ, γ, and μ, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

The term "antibody" herein is used in the broadest sense and specifically covers monoclonal antibodies (including full length monoclonal antibodies), polyclonal antibodies, multispecific antibodies (*e.g.*, bispecific antibodies), and antibody fragments so long as they exhibit the desired biological activity.

"Antibody fragments" comprise a portion of a full length antibody, generally the antigen binding or variable domain thereof. Examples of antibody fragments include Fab, Fab', $F(ab')_2$, and Fv fragments; diabodies; linear antibodies; single-chain antibody molecules; and multispecific antibodies formed from antibody fragments.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible naturally occurring mutations that

may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler *et al., Nature* 256:495 (1975), or may be made by recombinant DNA methods (see, *e.g.*, U.S. Patent No. 4,816,567). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson *et al., Nature* 352:624-628 (1991) and Marks *et al., J. Mol. Biol.* 222:581-597 (1991), for example.

The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Patent No. 4,816,567; and Morrison *et al., Proc. Natl. Acad. Sci. USA* 81:6851-6855 (1984)).

"Humanized" forms of non-human (*e.g.*, murine) antibodies are chimeric antibodies which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which hypervariable region residues of the recipient are replaced by hypervariable region residues from a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues which are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the

hypervariable regions correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones *et al.*, *Nature* 321:522-525 (1986); Reichmann *et al.*, *Nature* 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.* 2:593-596 (1992).

"Single-chain Fv" or "sFv" antibody fragments comprise the V_H and V_L domains of antibody, wherein these domains are present in a single polypeptide chain. Generally, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the sFv to form the desired structure for antigen binding. For a review of sFv see Pluckthun in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenburg and Moore eds. Springer-Verlag, New York, pp. 269-315 (1994).

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy chain variable domain (V_H) connected to a light chain variable domain (V_L) in the same polypeptide chain (V_H - V_L). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger *et al.*, *Proc. Natl. Acad. Sci. USA* 90:6444-6448 (1993).

The expression "linear antibodies" when used throughout this application refers to the antibodies described in Zapata *et al. Protein Eng.* 8(10):1057-1062 (1995). Briefly, these antibodies comprise a pair of tandem Fd segments (V_H-C_H1-V_H-C_H1) which form a pair of antigen binding regions. Linear antibodies can be bispecific or monospecific.

A "variant" anti-VEGF antibody, refers herein to a molecule which differs in amino acid sequence from a "parent" anti-VEGF antibody amino acid sequence by virtue of addition, deletion and/or substitution of one or more amino acid residue(s) in the parent antibody sequence. In the preferred embodiment, the variant comprises one or more amino acid substitution(s) in one or more hypervariable region(s) of the parent antibody. For example, the variant may comprise at least one, *e.g.* from about one to about ten, and preferably from about two to about five, substitutions in one or more hypervariable regions of the parent antibody. Ordinarily, the variant will have an amino acid sequence having at least 75% amino acid sequence identity with the parent antibody heavy or light chain variable

domain sequences (*e.g.* as in SEQ ID NO:7 or 8), more preferably at least 80%, more preferably at least 85%, more preferably at least 90%, and most preferably at least 95%. Identity or homology with respect to this sequence is defined herein as the percentage of amino acid residues in the candidate sequence that are identical with the parent antibody residues, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity. None of N-terminal, C-terminal, or internal extensions, deletions, or insertions into the antibody sequence shall be construed as affecting sequence identity or homology. The variant retains the ability to bind human VEGF and preferably has properties which are superior to those of the parent antibody. For example, the variant may have a stronger binding affinity, enhanced ability to inhibit VEGF-induced proliferation of endothelial cells and/or increased ability to inhibit VEGF-induced angiogenesis *in vivo*. To analyze such properties, one should compare a Fab form of the variant to a Fab form of the parent antibody or a full length form of the variant to a full length form of the parent antibody, for example, since it has been found that the format of the anti-VEGF antibody impacts its activity in the biological activity assays disclosed herein. The variant antibody of particular interest herein is one which displays at least about 10 fold, preferably at least about 20 fold, and most preferably at least about 50 fold, enhancement in biological activity when compared to the parent antibody.

The "parent" antibody herein is one which is encoded by an amino acid sequence used for the preparation of the variant. Preferably, the parent antibody has a human framework region and, if present, has human antibody constant region(s). For example, the parent antibody may be a humanized or human antibody.

An "isolated" antibody is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In preferred embodiments, the antibody will be purified (1) to greater than 95% by weight of antibody as determined by the Lowry method, and most preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using Coomassie blue or, preferably, silver stain. Isolated antibody includes the antibody *in situ* within recombinant

cells since at least one component of the antibody's natural environment will not be present. Ordinarily, however, isolated antibody will be prepared by at least one purification step.

The term "epitope tagged" when used herein refers to the anti-VEGF antibody fused to an "epitope tag". The epitope tag polypeptide has enough residues to provide an epitope against which an antibody thereagainst can be made, yet is short enough such that it does not interfere with activity of the VEGF antibody. The epitope tag preferably is sufficiently unique so that the antibody thereagainst does not substantially cross-react with other epitopes. Suitable tag polypeptides generally have at least 6 amino acid residues and usually between about 8-50 amino acid residues (preferably between about 9-30 residues). Examples include the flu HA tag polypeptide and its antibody 12CA5 (Field *et al. Mol. Cell. Biol.* 8:2159-2165 (1988)); the c-myc tag and the 8F9, 3C7, 6E10, G4, B7 and 9E10 antibodies thereto (Evan *et al., Mol. Cell. Biol.* 5(12):3610-3616 (1985)); and the Herpes Simplex virus glycoprotein D (gD) tag and its antibody (Paborsky *et al., Protein Engineering* 3(6):547-553 (1990)). In certain embodiments, the epitope tag is a "salvage receptor binding epitope". As used herein, the term "salvage receptor binding epitope" refers to an epitope of the Fc region of an IgG molecule (*e.g.*, IgG_1, IgG_2, IgG_3, or IgG_4) that is responsible for increasing the *in vivo* serum half-life of the IgG molecule.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (*e.g.*, I^{131}, I^{125}, Y^{90} and Re^{186}), chemotherapeutic agents, and toxins such as enzymatically active toxins of bacterial, fungal, plant or animal origin, or fragments thereof.

A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include Adriamycin, Doxorubicin, 5-Fluorouracil, Cytosine arabinoside ("Ara-C"), Cyclophosphamide, Thiotepa, Taxotere (docetaxel), Busulfan, Cytoxin, Taxol, Methotrexate, Cisplatin, Melphalan, Vinblastine, Bleomycin, Etoposide, Ifosfamide, Mitomycin C, Mitoxantrone, Vincreistine, Vinorelbine, Carboplatin, Teniposide, Daunomycin, Carminomycin, Aminopterin, Dactinomycin, Mitomycins, Esperamicins (see U.S. Pat. No. 4,675,187), Melphalan and other related nitrogen mustards.

The term "prodrug" as used in this application refers to a precursor or derivative form of a pharmaceutically active substance that is less cytotoxic to tumor cells compared

to the parent drug and is capable of being enzymatically activated or converted into the more active parent form. See, *e.g.*, Wilman, "Prodrugs in Cancer Chemotherapy" *Biochemical Society Transactions*, 14, pp. 375-382, 615th Meeting Belfast (1986) and Stella *et al.*, "Prodrugs: A Chemical Approach to Targeted Drug Delivery," *Directed Drug Delivery*, Borchardt *et al.*, (ed.), pp. 247-267, Humana Press (1985). The prodrugs of this invention include, but are not limited to, phosphate-containing prodrugs, thiophosphate-containing prodrugs, sulfate-containing prodrugs, peptide-containing prodrugs, D-amino acid-modified prodrugs, glycosylated prodrugs, β-lactam-containing prodrugs, optionally substituted phenoxyacetamide-containing prodrugs or optionally substituted phenylacetamide-containing prodrugs, 5-fluorocytosine and other 5-fluorouridine prodrugs which can be converted into the more active cytotoxic free drug. Examples of cytotoxic drugs that can be derivatized into a prodrug form for use in this invention include, but are not limited to, those chemotherapeutic agents described above.

The word "label" when used herein refers to a detectable compound or composition which is conjugated directly or indirectly to the antibody. The label may itself be detectable by itself (*e.g.*, radioisotope labels or fluorescent labels) or, in the case of an enzymatic label, may catalyze chemical alteration of a substrate compound or composition which is detectable.

By "solid phase" is meant a non-aqueous matrix to which the antibody of the present invention can adhere. Examples of solid phases encompassed herein include those formed partially or entirely of glass (*e.g.* controlled pore glass), polysaccharides (*e.g.*, agarose), polyacrylamides, polystyrene, polyvinyl alcohol and silicones. In certain embodiments, depending on the context, the solid phase can comprise the well of an assay plate; in others it is a purification column (*e.g.* an affinity chromatography column). This term also includes a discontinuous solid phase of discrete particles, such as those described in U.S. Patent No. 4,275,149.

A "liposome" is a small vesicle composed of various types of lipids, phospholipids and/or surfactant which is useful for delivery of a drug (such as the anti-VEGF antibodies disclosed herein and, optionally, a chemotherapeutic agent) to a mammal. The components of the liposome are commonly arranged in a bilayer formation, similar to the lipid arrangement of biological membranes. An "isolated" nucleic acid molecule is a nucleic acid molecule that is identified and separated from at least one contaminant nucleic acid molecule with which it is ordinarily associated in the natural source of the antibody nucleic acid. An

isolated nucleic acid molecule is other than in the form or setting in which it is found in nature. Isolated nucleic acid molecules therefore are distinguished from the nucleic acid molecule as it exists in natural cells. However, an isolated nucleic acid molecule includes a nucleic acid molecule contained in cells that ordinarily express the antibody where, for example, the nucleic acid molecule is in a chromosomal location different from that of natural cells.

The expression "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

As used herein, the expressions "cell," "cell line," and "cell culture" are used interchangeably and all such designations include progeny. Thus, the words "transformants" and "transformed cells" include the primary subject cell and cultures derived therefrom without regard for the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Mutant progeny that have the same function or biological activity as screened for in the originally transformed cell are included. Where distinct designations are intended, it will be clear from the context.

II. Modes for Carrying out the Invention

The examples hereinbelow describe the production of humanized and variant anti-

VEGF antibodies with desirable properties from a therapeutic perspective including: (a) strong binding affinity for the VEGF antigen; (b) an ability to inhibit VEGF-induced proliferation of endothelial cells *in vitro;* and (c) the ability to inhibit VEGF-induced angiogenesis *in vivo*.

Antibody affinities may be determined as described in the examples hereinbelow. Preferred humanized or variant antibodies are those which bind human VEGF with a K_d value of no more than about 1 x 10^{-7}M; preferably no more than about 1 x 10^{8} M; and most preferably no more than about 5 x 10^{-9}M.

Aside from antibodies with strong binding affinity for human VEGF, it is also desirable to select humanized or variant antibodies which have other beneficial properties from a therapeutic perspective. For example, the antibody may be one which inhibits endothelial cell growth in response to VEGF. In one embodiment, the antibody may be able to inhibit bovine capillary endothelial cell proliferation in response to a near maximally effective concentration of VEGF (3 ng/ml). Preferably, the antibody has an effective dose 50 (ED50) value of no more than about 5nM, preferably no more than about 1nM, and most preferably no more than about 0.5nM, for inhibiting VEGF-induced proliferation of endothelial cells in this "endothelial cell growth assay", *i.e.*, at these concentrations the antibody is able to inhibit VEGF-induced endothelial cell growth *in vitro* by 50%. A preferred "endothelial cell growth assay" involves culturing bovine adrenal cortex-derived capillary endothelial cells in the presence of low glucose Dulbecco's modified Eagle's medium (DMEM) (GIBCO) supplemented with 10% calf serum, 2 mM glutamine, and antibiotics (growth medium), essentially as described in Example 1 below. These endothelial cells are seeded at a density of 6 x 10^{3} cells per well, in 6-well plates in growth medium. Either parent anti-VEGF antibody (control), humanized or variant anti-VEGF antibody is then added at concentrations ranging between 1 and 5000 ng/ml. After 2-3 hr, purified VEGF was added to a final concentration of 3 ng/ml. For specificity control, each antibody may be added to endothelial cells at the concentration of 5000 ng/ml, either alone or in the presence of 2 ng/ml bFGF. After five or six days, cells are dissociated by exposure to trypsin and counted in a Coulter counter (Coulter Electronics, Hialeah, FL). Data may be analyzed by a four-parameter curve fitting program (KaleidaGraph).

The preferred humanized or variant anti-VEGF antibody may also be one which has *in vivo* tumor suppression activity. For example, the antibody may suppress the growth of

human A673 rhabdomyosarcoma cells or breast carcinoma MDA-MB-435 cells in nude mice. For *in vivo* tumor studies, human A673 rhabdomyosarcoma cells (ATCC; CRL 1598) or MDA-MB-435 cells (available from the ATCC) are cultured in DMEM/F12 supplemented with 10% fetal bovine serum, 2 mM glutamine and antibiotics as described in Example 1 below. Female BALB/c nude mice, 6-10 weeks old, are injected subcutaneously with 2 x 10^6 tumor cells in the dorsal area in a volume of 200 µl. Animals are then treated with the humanized or variant antibody and a control antibody with no activity in this assay. The humanized or variant anti-VEGF MAb is administered at a dose of 0.5 and/or 5 mg/kg. Each MAb is administered twice weekly intra peritoneally in a volume of 100 µl, starting 24 hr after tumor cell inoculation. Tumor size is determined at weekly intervals. Four weeks after tumor cell inoculation, animals are euthanized and the tumors are removed and weighed. Statistical analysis may be performed by ANOVA. Preferably, the antibody in this "*in vivo* tumor assay" inhibits about 50-100%, preferably about 70-100% and most preferably about 80-100% human A673 tumor cell growth at a dose of 5mg/kg.

In the preferred embodiment, the humanized or variant antibody fails to elicit an immunogenic response upon administration of a therapeutically effective amount of the antibody to a human patient. If an immunogenic response is elicited, preferably the response will be such that the antibody still provides a therapeutic benefit to the patient treated therewith.

The humanized or variant antibody is also preferably one which is able to inhibit VEGF-induced angiogenesis in a human, *e.g.* to inhibit human tumor growth and/or inhibit intraocular angiogenesis in retinal disorders.

Preferred antibodies bind the "epitope A4.6.1" as herein defined. To screen for antibodies which bind to the epitope on human VEGF bound by an antibody of interest (*e.g.*, those which block binding of the A4.6.1 antibody to human VEGF), a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping, *e.g.* as described in Champe *et al., J. Biol. Chem.* 270:1388-1394 (1995), can be performed to determine whether the antibody binds an epitope of interest.

The antibodies of the preferred embodiment herein have a heavy chain variable domain comprising an amino acid sequence represented by the formula: FR1-CDRH1-FR2-CDRH2-FR3-CDRH3-FR4, wherein "FR1-4" represent the four framework regions and

"CDRH1-3" represent the three hypervariable regions of an anti-VEGF antibody variable heavy domain. FR1-4 may be derived from a "consensus sequence" (*i.e.* the most common amino acids of a class, subclass or subgroup of heavy or light chains of human immunoglobulins) as in the examples below or may be derived from an individual human antibody framework region or from a combination of different framework region sequences. Many human antibody framework region sequences are compiled in Kabat *et al.*, *supra*, for example. In one preferred embodiment, the variable heavy FR is provided by a consensus sequence of a human immunoglobulin subgroup as compiled by Kabat *et al.*, *supra*. Preferably, the human immunoglobulin subgroup is human heavy chains subgroup III (*e.g.* as in SEQ ID NO:11).

The human variable heavy FR sequence preferably has substitutions therein, *e.g.* wherein the human FR residue is replaced by a corresponding nonhuman residue (by "corresponding nonhuman residue" is meant the nonhuman residue with the same Kabat positional numbering as the human residue of interest when the human and nonhuman sequences are aligned), but replacement with the nonhuman residue is not necessary. For example, a replacement FR residue other than the corresponding nonhuman residue may be selected by phage display (see Example 2 below). Exemplary variable heavy FR residues which may be substituted include any one or more of FR residue numbers: 37H, 49H, 67H, 69H, 71H, 73H, 75H, 76H, 78H, 94H (Kabat residue numbering employed here). Preferably at least two, or at least three, or at least four of these residues are substituted. A particularly preferred combination of FR substitutions is: 49H, 69H, 71H, 73H, 76H, 78H, and 94H.

With respect to the heavy chain hypervariable regions, these preferably have amino acid sequences as follows:

CDRH1

$GYX_1X_2X_3X_4YGX_5N$ (SEQ ID NO:117), wherein X_1 is D, T or E, but preferably is D or T; X_2 is F, W, or Y, but preferably is F; X_3 is T, Q, G or S, but preferably is T; X_4 is H or N; and X_5 is M or I, but preferably is M.

CDRH2

$WINTX_1TGEPTYAADFKR$ (SEQ ID NO:118), wherein X_1 is Y or W, but preferably is Y.

CDRH3

$YPX_1YX_2X_3X_4X_5HWYFDV$ (SEQ ID NO:119), wherein X_1 is H or Y; X_2 is Y, R, K, I, T, E, or W, but preferably is Y; X_3 is G, N, A, D, Q, E, T, K, or S, but preferably is G; X_4 is S, T, K, Q, N, R, A, E, or G, but preferably is S or T; and X_5 is S or G, but preferably is S.

The heavy chain variable domain optionally comprises what has been designated "CDR7" herein within (*i.e.* forming part of) FR3 (see Figs. 9B and 10B), wherein CDR7 may have the following amino acid sequence:

CDR7

$X_1SX_2DX_3X_4X_5X_6TX_7$ (SEQ ID NO:120), wherein X_1 is F, I , V, L, or A, but preferably is F; X_2 is A, L, V, or I, but preferably is L; X_3 is T, V or K, but preferably is T; X_4 is S or W, but preferably is S; X_5 is S, or K, but preferably is K; X_6 is N, or S, but preferably is S; and X_7 is V, A, L or I, but preferably is A.

The antibodies of the preferred embodiment herein have a light chain variable domain comprising an amino acid sequence represented by the formula: FR1-CDRL1-FR2-CDRL2-FR3-CDRL3-FR4, wherein "FR1-4" represent the four framework regions and "CDRL1-3" represent the three hypervariable regions of an anti-VEGF antibody variable heavy domain. FR1-4 may be derived from a "consensus sequence" (*i.e.* the most common amino acids of a class, subclass or subgroup of heavy or light chains of human immunoglobulins) as in the examples below or may be derived from an individual human antibody framework region or from a combination of different framework region sequences. In one preferred embodiment, the variable light FR is provided by a consensus sequence of a human immunoglobulin subgroup as compiled by Kabat *et al.*, *supra*. Preferably, the human immunoglobulin subgroup is human kappa light chains subgroup I (*e.g.* as in SEQ ID NO:12).

The human variable light FR sequence preferably has substitutions therein, *e.g.* wherein the human FR residue is replaced by a corresponding mouse residue, but replacement with the nonhuman residue is not necessary. For example, a replacement residue other than the corresponding nonhuman residue may be selected by phage display (see Example 2 below). Exemplary variable light FR residues which may be substituted include any one or more of FR residue numbers: 4L, 46L and 71L (Kabat residue numbering employed here).

Preferably only 46L is substituted. In another embodiment, both 4L and 46L are substituted.

With respect to the CDRs, these preferably have amino acid sequences as follows:

CDRL1

$X_1AX_2X_3X_4X_5SNYLN$ (SEQ ID NO:121), wherein X_1 is R or S, but preferably is S; X_2 is S or N, but preferably is S; X_3 is Q or E, but preferably is Q; X_4 is Q or D, but preferably is D; and X_5 is I or L, but preferably is I.

CDRL2

FTSSLHS (SEQ ID NO:122).

CDRL3

$QQYSX_1X_2PWT$ (SEQ ID NO:123), wherein X_1 is T, A or N, but preferably is T; and X_2 is V or T, but preferably is V.

Preferred humanized anti-VEGF antibodies are those having the heavy and/or light variable domain sequences of F(ab)-12 in Example 1 and variants thereof such as affinity matured forms including variants Y0317, Y0313-1 and Y0238-3 in Example 3, with Y0317 being the preferred variant. Methods for generating humanized anti-VEGF antibodies of interest herein are elaborated in more detail below.

A. Antibody Preparation

Methods for humanizing nonhuman VEGF antibodies and generating variants of anti-VEGF antibodies are described in the examples below. In order to humanize an anti-VEGF antibody, the nonhuman antibody starting material is prepared. Where a variant is to be generated, the parent antibody is prepared. Exemplary techniques for generating such nonhuman antibody starting material and parent antibodies will be described in the following sections.

(i) Antigen preparation

The VEGF antigen to be used for production of antibodies may be, *e.g.*, intact VEGF or a fragment of VEGF (*e.g.* a VEGF fragment comprising "epitope A4.6.1"). Other forms of VEGF useful for generating antibodies will be apparent to those skilled in the art. The VEGF antigen used to generate the antibody, is preferably human VEGF, *e.g.* as described in Leung *et al.*, *Science* 246:1306 (1989), and Houck *et al.*, *Mol. Endocrin.* 5:1806 (1991).

(ii) Polyclonal antibodies

Polyclonal antibodies are preferably raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the relevant antigen to a protein that is immunogenic in the species to be immunized, *e.g.*, keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, $SOCl_2$, or $R^1N=C=NR$, where R and R^1 are different alkyl groups.

Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining, *e.g.*, 100 µg or 5 µg of the protein or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with 1/5 to 1/10 the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

(iii) Monoclonal antibodies

Monoclonal antibodies may be made using the hybridoma method first described by Kohler *et al., Nature*, 256:495 (1975), or may be made by recombinant DNA methods (U.S. Patent No. 4,816,567).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster or macaque monkey, is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized *in vitro*. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59-103 (Academic Press, 1986)).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOP-21 and M.C.-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, California USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Maryland USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.*, 133:3001 (1984); Brodeur *et al.*, *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987)).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson *et al.*, *Anal. Biochem.*, 107:220 (1980).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59-103 (Academic Press, 1986)). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown *in vivo* as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel

electrophoresis, dialysis, or affinity chromatography.

DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (*e.g.*, by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the monoclonal antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Recombinant production of antibodies will be described in more detail below.

(iv) Humanization and amino acid sequence variants

Examples 1-2 below describe procedures for humanization of an anti-VEGF antibody. In certain embodiments, it may be desirable to generate amino acid sequence variants of these humanized antibodies, particularly where these improve the binding affinity or other biological properties of the humanized antibody. Example 3 describes methodologies for generating amino acid sequence variants of an anti-VEGF antibody with enhanced affinity relative to the parent antibody.

Amino acid sequence variants of the anti-VEGF antibody are prepared by introducing appropriate nucleotide changes into the anti-VEGF antibody DNA, or by peptide synthesis. Such variants include, for example, deletions from, and/or insertions into and/or substitutions of, residues within the amino acid sequences of the anti-VEGF antibodies of the examples herein. Any combination of deletion, insertion, and substitution is made to arrive at the final construct, provided that the final construct possesses the desired characteristics. The amino acid changes also may alter post-translational processes of the humanized or variant anti-VEGF antibody, such as changing the number or position of glycosylation sites.

A useful method for identification of certain residues or regions of the anti-VEGF antibody that are preferred locations for mutagenesis is called "alanine scanning mutagenesis," as described by Cunningham and Wells *Science*, 244:1081-1085 (1989). Here, a residue or group of target residues are identified (*e.g.*, charged residues such as arg, asp, his, lys, and glu) and replaced by a neutral or negatively charged amino acid (most preferably alanine or polyalanine) to affect the interaction of the amino acids with VEGF antigen. Those amino acid locations demonstrating functional sensitivity to the substitutions then are refined

by introducing further or other variants at, or for, the sites of substitution. Thus, while the site for introducing an amino acid sequence variation is predetermined, the nature of the mutation *per se* need not be predetermined. For example, to analyze the performance of a mutation at a given site, ala scanning or random mutagenesis is conducted at the target codon or region and the expressed anti-VEGF antibody variants are screened for the desired activity. Alanine scanning mutagenesis is described in Example 3.

Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Examples of terminal insertions include an anti-VEGF antibody with an N-terminal methionyl residue or the antibody fused to an epitope tag. Other insertional variants of the anti-VEGF antibody molecule include the fusion to the N- or C-terminus of the anti-VEGF antibody of an enzyme or a polypeptide which increases the serum half-life of the antibody (see below).

Another type of variant is an amino acid substitution variant. These variants have at least one amino acid residue in the anti-VEGF antibody molecule removed and a different residue inserted in its place. The sites of greatest interest for substitutional mutagenesis include the hypervariable regions, but FR alterations are also contemplated. Conservative substitutions are shown in Table 1 under the heading of "preferred substitutions". If such substitutions result in a change in biological activity, then more substantial changes, denominated "exemplary substitutions" in Table 1, or as further described below in reference to amino acid classes, may be introduced and the products screened.

Table 1

Original Residue	Exemplary Substitutions	Preferred Substitutions
Ala (A)	val; leu; ile	val
Arg (R)	lys; gln; asn	lys
Asn (N)	gln; his; asp, lys; arg	gln
Asp (D)	glu; asn	glu
Cys (C)	ser; ala	ser
Gln (Q)	asn; glu	asn
Glu (E)	asp; gln	asp

Original Residue	Exemplary Substitutions	Preferred Substitutions
Gly (G)	ala	ala
His (H)	asn; gln; lys; arg	arg
Ile (I)	leu; val; met; ala; phe; norleucine	leu
Leu (L)	norleucine; ile; val; met; ala; phe	ile
Lys (K)	arg; gln; asn	arg
Met (M)	leu; phe; ile	leu
Phe (F)	leu; val; ile; ala; tyr	tyr
Pro (P)	ala	ala
Ser (S)	thr	thr
Thr (T)	ser	ser
Trp (W)	tyr; phe	tyr
Tyr (Y)	trp; phe; thr; ser	phe
Val (V)	ile; leu; met; phe; ala; norleucine	leu

Substantial modifications in the biological properties of the antibody are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Naturally occurring residues are divided into groups based on common side-chain properties:

(1) hydrophobic: norleucine, met, ala, val, leu, ile;

(2) neutral hydrophilic: cys, ser, thr;

(3) acidic: asp, glu;

(4) basic: asn, gln, his, lys, arg;

(5) residues that influence chain orientation: gly, pro; and

(6) aromatic: trp, tyr, phe.

Non-conservative substitutions will entail exchanging a member of one of these classes for another class.

Any cysteine residue not involved in maintaining the proper conformation of the humanized or variant anti-VEGF antibody also may be substituted, generally with serine, to improve the oxidative stability of the molecule and prevent aberrant crosslinking. Conversely, cysteine bond(s) may be added to the antibody to improve its stability (particularly where the antibody is an antibody fragment such as an Fv fragment).

A particularly preferred type of substitutional variant involves substituting one or more hypervariable region residues of a parent antibody (*e.g.* a humanized or human antibody). Generally, the resulting variant(s) selected for further development will have improved biological properties relative to the parent antibody from which they are generated. A convenient way for generating such substitutional variants is affinity maturation using phage display (see Example 3 herein). Briefly, several hypervariable region sites (*e.g.* 6-7 sites) are mutated to generate all possible amino substitutions at each site. The antibody variants thus generated are displayed in a monovalent fashion from filamentous phage particles as fusions to the gene III product of M13 packaged within each particle. The phage-displayed variants are then screened for their biological activity (*e.g.* binding affinity) as herein disclosed. In order to identify candidate hypervariable region sites for modification, alanine scanning mutagenesis (see Example 3) can be performed to identified hypervariable region residues contributing significantly to antigen binding. Alternatively, or in addition, it may be beneficial to analyze a crystal structure of the antigen-antibody complex to identify contact points between the antibody and human VEGF. Such contact residues and neighboring residues are candidates for substitution according to the techniques elaborated herein. Once such variants are generated, the panel of variants is subjected to screening as described herein and antibodies with superior properties in one or more relevant assays may be selected for further development.

Another type of amino acid variant of the antibody alters the original glycosylation pattern of the antibody. By altering is meant deleting one or more carbohydrate moieties found in the antibody, and/or adding one or more glycosylation sites that are not present in the antibody.

Glycosylation of antibodies is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The

tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-aceylgalactosamine, galactose, or xylose to a hydroxyamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

Addition of glycosylation sites to the antibody is conveniently accomplished by altering the amino acid sequence such that it contains one or more of the above-described tripeptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the sequence of the original antibody (for O-linked glycosylation sites).

Nucleic acid molecules encoding amino acid sequence variants of the anti-VEGF antibody are prepared by a variety of methods known in the art. These methods include, but are not limited to, isolation from a natural source (in the case of naturally occurring amino acid sequence variants) or preparation by oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared variant or a non-variant version of the anti-VEGF antibody.

(v) Human antibodies

As an alternative to humanization, human antibodies can be generated. For example, it is now possible to produce transgenic animals (*e.g.*, mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, *e.g.*, Jakobovits *et al., Proc. Natl. Acad. Sci. USA*, 90:2551 (1993); Jakobovits *et al., Nature*, 362:255-258 (1993); Bruggermann *et al., Year in Immuno.*, 7:33 (1993); and US Patents 5,591,669, 5,589,369 and 5,545,807. Human antibodies can also be derived from phage-display libraries (Hoogenboom *et al., J. Mol. Biol.*, 227:381 (1991); Marks *et al., J. Mol. Biol.*, 222:581-597 (1991); and US Patents 5,565,332 and 5,573,905). As discussed above,

human antibodies may also be generated by *in vitro* activated B cells (see US Patents 5,567,610 and 5,229,275)

(vi) Antibody fragments

In certain embodiments, the humanized or variant anti-VEGF antibody is an antibody fragment. Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, *e.g.*, Morimoto *et al.*, *Journal of Biochemical and Biophysical Methods* 24:107-117 (1992) and Brennan *et al.*, *Science* 229:81 (1985)). However, these fragments can now be produced directly by recombinant host cells. For example, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form $F(ab')_2$ fragments (Carter *et al.*, *Bio/Technology* 10:163-167 (1992)). In another embodiment, the $F(ab')_2$ is formed using the leucine zipper GCN4 to promote assembly of the $F(ab')_2$ molecule. According to another approach, Fv, Fab or $F(ab')_2$ fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner.

(vii) Multispecific antibodies

In some embodiments, it may be desirable to generate multispecific (*e.g.* bispecific) humanized or variant anti-VEGF antibodies having binding specificities for at least two different epitopes. Exemplary bispecific antibodies may bind to two different epitopes of the VEGF protein. Alternatively, an anti-VEGF arm may be combined with an arm which binds to a triggering molecule on a leukocyte such as a T-cell receptor molecule (*e.g.*, CD2 or CD3), or Fc receptors for IgG (FcγR), such as FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) so as to focus cellular defense mechanisms to the VEGF-expressing cell. Bispecific antibodies may also be used to localize cytotoxic agents to cells which express VEGF. These antibodies possess an VEGF-binding arm and an arm which binds the cytotoxic agent (*e.g.*, saporin, anti-interferon-α, vinca alkaloid, ricin A chain, methotrexate or radioactive isotope hapten). Bispecific antibodies can be prepared as full length antibodies or antibody fragments (*e.g.*, $F(ab')_2$ bispecific antibodies).

According to another approach for making bispecific antibodies, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C_H3 domain of an antibody constant domain. In this method,

one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (*e.g.*, tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (*e.g.*, alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers. See WO96/27011 published September 6, 1996.

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in US Patent No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan *et al.*, *Science* 229:81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate $F(ab')_2$ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes. In yet a further embodiment, Fab'-SH fragments directly recovered from *E. coli* can be chemically coupled *in vitro* to form bispecific antibodies. Shalaby *et al.*, *J. Exp. Med.* 175:217-225 (1992).

Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny *et al.*, *J. Immunol.* 148(5):1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers.

The "diabody" technology described by Hollinger *et al.*, *Proc. Natl. Acad. Sci. USA* 90:6444-6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber *et al.*, *J. Immunol.* 152:5368 (1994). Alternatively, the bispecific antibody may be a "linear antibody" produced as described in Zapata *et al. Protein Eng.* 8(10):1057-1062 (1995).

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt *et al.*, *J. Immunol.* 147:60 (1991).

(viii) Other modifications

Other modifications of the humanized or variant anti-VEGF antibody are contemplated. For example, it may be desirable to modify the antibody of the invention with respect to effector function, so as to enhance the effectiveness of the antibody in treating cancer, for example. For example cysteine residue(s) may be introduced in the Fc region, thereby allowing interchain disulfide bond formation in this region. The homodimeric antibody thus generated may have improved internalization capability and/or increased complement-mediated cell killing and antibody-dependent cellular cytotoxicity (ADCC). See Caron *et al.*, *J. Exp Med.* 176:1191-1195 (1992) and Shopes, B. *J. Immunol.* 148:2918-2922 (1992). Homodimeric antibodies with enhanced anti-tumor activity may also be prepared using heterobifunctional cross-linkers as described in Wolff *et al.*, *Cancer Research* 53:2560-2565 (1993). Alternatively, an antibody can be engineered which has dual Fc regions and may thereby have enhanced complement lysis and ADCC capabilities. See Stevenson *et al.*, *Anti-Cancer Drug Design* 3:219-230 (1989).

The invention also pertains to immunoconjugates comprising the antibody described herein conjugated to a cytotoxic agent such as a chemotherapeutic agent, toxin (*e.g.*, an enzymatically active toxin of bacterial, fungal, plant or animal origin, or fragments thereof), or a radioactive isotope (*i.e.*, a radioconjugate).

Chemotherapeutic agents useful in the generation of such immunoconjugates have been described above. Enzymatically active toxins and fragments thereof which can be used

include diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from *Pseudomonas aeruginosa*), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, *Aleurites fordii* proteins, dianthin proteins, *Phytolaca americana* proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin and the tricothecenes. A variety of radionuclides are available for the production of radioconjugated anti-VEGF antibodies. Examples include ^{212}Bi, ^{131}I, ^{131}In, ^{90}Y and ^{186}Re.

Conjugates of the antibody and cytotoxic agent are made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutareldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta *et al.*, *Science* 238:1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody. See WO94/11026.

In another embodiment, the antibody may be conjugated to a "receptor" (such streptavidin) for utilization in tumor pretargeting wherein the antibody-receptor conjugate is administered to the patient, followed by removal of unbound conjugate from the circulation using a clearing agent and then administration of a "ligand" (*e.g.*, avidin) which is conjugated to a cytotoxic agent (*e.g.*, a radionuclide).

The anti-VEGF antibodies disclosed herein may also be formulated as immunoliposomes. Liposomes containing the antibody are prepared by methods known in the art, such as described in Epstein *et al.*, *Proc. Natl. Acad. Sci. USA* 82:3688 (1985); Hwang *et al.*, *Proc. Natl Acad. Sci. USA* 77:4030 (1980); and U.S. Pat. Nos. 4,485,045 and 4,544,545. Liposomes with enhanced circulation time are disclosed in U.S. Patent No. 5,013,556.

Particularly useful liposomes can be generated by the reverse phase evaporation method with a lipid composition comprising phosphatidylcholine, cholesterol and PEG-derivatized phosphatidylethanolamine (PEG-PE). Liposomes are extruded through filters of

defined pore size to yield liposomes with the desired diameter. Fab' fragments of the antibody of the present invention can be conjugated to the liposomes as described in Martin *et al., J. Biol. Chem.* 257:286-288 (1982) via a disulfide interchange reaction. A chemotherapeutic agent (such as Doxorubicin) is optionally contained within the liposome. See Gabizon *et al., J. National Cancer Inst.* 81(19):1484 (1989)

The antibody of the present invention may also be used in ADEPT by conjugating the antibody to a prodrug-activating enzyme which converts a prodrug (*e.g.*, a peptidyl chemotherapeutic agent, see WO81/01145) to an active anti-cancer drug. See, for example, WO 88/07378 and U.S. Patent No. 4,975,278.

The enzyme component of the immunoconjugate useful for ADEPT includes any enzyme capable of acting on a prodrug in such a way so as to covert it into its more active, cytotoxic form.

Enzymes that are useful in the method of this invention include, but are not limited to, alkaline phosphatase useful for converting phosphate-containing prodrugs into free drugs; arylsulfatase useful for converting sulfate-containing prodrugs into free drugs; cytosine deaminase useful for converting non-toxic 5-fluorocytosine into the anti-cancer drug, 5-fluorouracil; proteases, such as serratia protease, thermolysin, subtilisin, carboxypeptidases and cathepsins (such as cathepsins B and L), that are useful for converting peptide-containing prodrugs into free drugs; D-alanylcarboxypeptidases, useful for converting prodrugs that contain D-amino acid substituents; carbohydrate-cleaving enzymes such as β-galactosidase and neuraminidase useful for converting glycosylated prodrugs into free drugs; β-lactamase useful for converting drugs derivatized with β-lactams into free drugs; and penicillin amidases, such as penicillin V amidase or penicillin G amidase, useful for converting drugs derivatized at their amine nitrogens with phenoxyacetyl or phenylacetyl groups, respectively, into free drugs. Alternatively, antibodies with enzymatic activity, also known in the art as "abzymes", can be used to convert the prodrugs of the invention into free active drugs (see, *e.g.*, Massey, *Nature* 328:457-458 (1987)). Antibody-abzyme conjugates can be prepared as described herein for delivery of the abzyme to a tumor cell population.

The enzymes of this invention can be covalently bound to the anti-VEGF antibodies by techniques well known in the art such as the use of the heterobifunctional crosslinking reagents discussed above. Alternatively, fusion proteins comprising at least the antigen binding region of an antibody of the invention linked to at least a functionally active portion

of an enzyme of the invention can be constructed using recombinant DNA techniques well known in the art (see, *e.g.*, Neuberger *et al.*, *Nature* 312:604-608 (1984)).

In certain embodiments of the invention, it may be desirable to use an antibody fragment, rather than an intact antibody, to increase tumor penetration, for example. In this case, it may be desirable to modify the antibody fragment in order to increase its serum half life. This may be achieved, for example, by incorporation of a salvage receptor binding epitope into the antibody fragment (*e.g.*, by mutation of the appropriate region in the antibody fragment or by incorporating the epitope into a peptide tag that is then fused to the antibody fragment at either end or in the middle, *e.g.*, by DNA or peptide synthesis). See WO96/32478 published October 17, 1996.

The salvage receptor binding epitope generally constitutes a region wherein any one or more amino acid residues from one or two loops of a Fc domain are transferred to an analogous position of the antibody fragment. Even more preferably, three or more residues from one or two loops of the Fc domain are transferred. Still more preferred, the epitope is taken from the CH2 domain of the Fc region (*e.g.*, of an IgG) and transferred to the CH1, CH3, or V_H region, or more than one such region, of the antibody. Alternatively, the epitope is taken from the CH2 domain of the Fc region and transferred to the C_L region or V_L region, or both, of the antibody fragment.

In one most preferred embodiment, the salvage receptor binding epitope comprises the sequence: PKNSSMISNTP (SEQ ID NO:17), and optionally further comprises a sequence selected from the group consisting of HQSLGTQ (SEQ ID NO:18), HQNLSDGK (SEQ ID NO:19), HQNISDGK (SEQ ID NO:20), or VISSHLGQ (SEQ ID NO:21), particularly where the antibody fragment is a Fab or $F(ab')_2$. In another most preferred embodiment, the salvage receptor binding epitope is a polypeptide containing the sequence(s): HQNLSDGK (SEQ ID NO:19), HQNISDGK (SEQ ID NO:20), or VISSHLGQ (SEQ ID NO:21) and the sequence: PKNSSMISNTP (SEQ ID NO:17).

Covalent modifications of the humanized or variant anti-VEGF antibody are also included within the scope of this invention. They may be made by chemical synthesis or by enzymatic or chemical cleavage of the antibody, if applicable. Other types of covalent modifications of the antibody are introduced into the molecule by reacting targeted amino acid residues of the antibody with an organic derivatizing agent that is capable of reacting with selected side chains or the N- or C-terminal residues. Exemplary covalent modifications

of polypeptides are described in US Patent 5,534,615, specifically incorporated herein by reference. A preferred type of covalent modification of the antibody comprises linking the antibody to one of a variety of nonproteinaceous polymers, *e.g.*, polyethylene glycol, polypropylene glycol, or polyoxyalkylenes, in the manner set forth in U.S. Patent Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

B. Vectors, Host Cells and Recombinant Methods

The invention also provides isolated nucleic acid encoding the humanized or variant anti-VEGF antibody, vectors and host cells comprising the nucleic acid, and recombinant techniques for the production of the antibody.

For recombinant production of the antibody, the nucleic acid encoding it may be isolated and inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. In another embodiment, the antibody may be produced by homologous recombination, *e.g.* as described in US Patent 5,204,244, specifically incorporated herein by reference. DNA encoding the monoclonal antibody is readily isolated and sequenced using conventional procedures (*e.g.*, by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the antibody). Many vectors are available. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence, *e.g.*, as described in US Patent 5,534,615 issued July 9, 1996 and specifically incorporated herein by reference.

Suitable host cells for cloning or expressing the DNA in the vectors herein are the prokaryote, yeast, or higher eukaryote cells described above. Suitable prokaryotes for this purpose include eubacteria, such as Gram-negative or Gram-positive organisms, for example, Enterobacteriaceae such as *Escherichia*, *e.g.*, *E. coli*, *Enterobacter*, *Erwinia*, *Klebsiella*, *Proteus*, *Salmonella*, *e.g.*, *Salmonella typhimurium*, *Serratia*, *e.g.*, *Serratia marcescans*, and *Shigella*, as well as *Bacilli* such as *B. subtilis* and *B. licheniformis* (*e.g.*, *B. licheniformis* 41P disclosed in DD 266,710 published 12 April 1989), *Pseudomonas* such as *P. aeruginosa*, and *Streptomyces*. One preferred *E. coli* cloning host is *E. coli* 294 (ATCC 31,446), although other strains such as *E. coli* B, *E. coli* X1776 (ATCC 31,537), and *E. coli* W3110 (ATCC 27,325) are suitable. These examples are illustrative rather than limiting.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for anti-VEGF antibody-encoding vectors.

Saccharomyces cerevisiae, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as *Schizosaccharomyces pombe*; *Kluyveromyces* hosts such as, *e.g.*, *K. lactis*, *K. fragilis* (ATCC 12,424), *K. bulgaricus* (ATCC 16,045), *K. wickeramii* (ATCC 24,178), *K. waltii* (ATCC 56,500), *K. drosophilarum* (ATCC 36,906), *K . thermotolerans*, and *K. marxianus; yarrowia* (EP 402,226); *Pichia pastoris* (EP 183,070); *Candida; Trichoderma reesia* (EP 244,234); *Neurospora crassa*; *Schwanniomyces* such as *Schwanniomyces occidentalis*; and filamentous fungi such as, *e.g.*, *Neurospora, Penicillium*, *Tolypocladium*, and *Aspergillus* hosts such as *A. nidulans* and *A. niger*.

Suitable host cells for the expression of glycosylated anti-VEGF antibody are derived from multicellular organisms. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* have been identified. A variety of viral strains for transfection are publicly available, *e.g.*, the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV, and such viruses may be used as the virus herein according to the present invention, particularly for transfection of *Spodoptera frugiperda* cells. Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can also be utilized as hosts.

However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure. Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham *et al., J. Gen Virol.* 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub *et al., Proc. Natl. Acad. Sci. USA* 77:4216 (1980)); mouse sertoli cells (TM4, Mather, *Biol. Reprod.* 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather *et al.*,

Annals N.Y. Acad. Sci. 383:44-68 (1982)); MRC 5 cells; FS4 cells; and a human hepatoma line (Hep G2).

Host cells are transformed with the above-described expression or cloning vectors for anti-VEGF antibody production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

The host cells used to produce the anti-VEGF antibody of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ((MEM), (Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ((DMEM), Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham *et al.*, *Meth. Enz.* 58:44 (1979), Barnes *et al.*, *Anal. Biochem.*102:255 (1980), U.S. Pat. Nos. 4,767,704; 4,657,866; 4,927,762; 4,560,655; or 5,122,469; WO 90/03430; WO 87/00195; or U.S. Patent Re. 30,985 may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleotides (such as adenosine and thymidine), antibiotics (such as GENTAMYCIN™drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

When using recombinant techniques, the antibody can be produced intracellularly, in the periplasmic space, or directly secreted into the medium. If the antibody is produced intracellularly, as a first step, the particulate debris, either host cells or lysed fragments, is removed, for example, by centrifugation or ultrafiltration. Carter *et al.*, *Bio/Technology* 10:163-167 (1992) describe a procedure for isolating antibodies which are secreted to the periplasmic space of *E. coli*. Briefly, cell paste is thawed in the presence of sodium acetate (pH 3.5), EDTA, and phenylmethylsulfonylfluoride (PMSF) over about 30 min. Cell debris can be removed by centrifugation. Where the antibody is secreted into the medium, supernatants from such expression systems are generally first concentrated using a

commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. A protease inhibitor such as PMSF may be included in any of the foregoing steps to inhibit proteolysis and antibiotics may be included to prevent the growth of adventitious contaminants.

The antibody composition prepared from the cells can be purified using, for example, hydroxylapatite chromatography, gel electrophoresis, dialysis, and affinity chromatography, with affinity chromatography being the preferred purification technique. The suitability of protein A as an affinity ligand depends on the species and isotype of any immunoglobulin Fc domain that is present in the antibody. Protein A can be used to purify antibodies that are based on human γ1, γ2, or γ4 heavy chains (Lindmark *et al.*, *J. Immunol. Meth.* 62:1-13 (1983)). Protein G is recommended for all mouse isotypes and for human γ3 (Guss *et al.*, *EMBO J.* 5:15671575 (1986)). The matrix to which the affinity ligand is attached is most often agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or poly(styrenedivinyl)benzene allow for faster flow rates and shorter processing times than can be achieved with agarose. Where the antibody comprises a C_H3 domain, the Bakerbond ABX™resin (J. T. Baker, Phillipsburg, NJ) is useful for purification. Other techniques for protein purification such as fractionation on an ion-exchange column, ethanol precipitation, Reverse Phase HPLC, chromatography on silica, chromatography on heparin SEPHAROSE™ chromatography on an anion or cation exchange resin (such as a polyaspartic acid column), chromatofocusing, SDS-PAGE, and ammonium sulfate precipitation are also available depending on the antibody to be recovered.

Following any preliminary purification step(s), the mixture comprising the antibody of interest and contaminants may be subjected to low pH hydrophobic interaction chromatography using an elution buffer at a pH between about 2.5-4.5, preferably performed at low salt concentrations (*e.g.*, from about 0-0.25M salt).

C. Pharmaceutical Formulations

Therapeutic formulations of the antibody are prepared for storage by mixing the antibody having the desired degree of purity with optional physiologically acceptable carriers, excipients or stabilizers (*Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980)), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids;

antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (*e.g.*, Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG).

The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other (see Section F below). Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

The active ingredients may also be entrapped in microcapsule prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsule and poly-(methylmethacylate) microcapsule, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980).

The formulations to be used for *in vivo* administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes.

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, *e.g.*, films, or microcapsule. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the Lupron Depot™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and

leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated antibodies remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37°C, resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S-S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

D. Non-therapeutic Uses for the Antibody

The antibodies of the invention may be used as affinity purification agents. In this process, the antibodies are immobilized on a solid phase such a Sephadex resin or filter paper, using methods well known in the art. The immobilized antibody is contacted with a sample containing the VEGF protein (or fragment thereof) to be purified, and thereafter the support is washed with a suitable solvent that will remove substantially all the material in the sample except the VEGF protein, which is bound to the immobilized antibody. Finally, the support is washed with another suitable solvent, such as glycine buffer, pH 5.0, that will release the VEGF protein from the antibody.

Anti-VEGF antibodies may also be useful in diagnostic assays for VEGF protein, *e.g.*, detecting its expression in specific cells, tissues, or serum. Such diagnostic methods may be useful in cancer diagnosis.

For diagnostic applications, the antibody typically will be labeled with a detectable moiety. Numerous labels are available which can be generally grouped into the following categories:

(a) Radioisotopes, such as ^{35}S, ^{14}C, ^{125}I, ^{3}H, and ^{131}I. The antibody can be labeled with the radioisotope using the techniques described in *Current Protocols in Immunology*, Volumes 1 and 2, Coligen *et al.*, Ed. Wiley-Interscience, New York, New York, Pubs. (1991) for example and radioactivity can be measured using scintillation counting.

(b) Fluorescent labels such as rare earth chelates (europium chelates) or fluorescein and its derivatives, rhodamine and its derivatives, dansyl, Lissamine, phycoerythrin and Texas

Red are available. The fluorescent labels can be conjugated to the antibody using the techniques disclosed in *Current Protocols in Immunology*, *supra*, for example. Fluorescence can be quantified using a fluorimeter.

(c) Various enzyme-substrate labels are available and U.S. Patent No. 4,275,149 provides a review of some of these. The enzyme generally catalyzes a chemical alteration of the chromogenic substrate which can be measured using various techniques. For example, the enzyme may catalyze a color change in a substrate, which can be measured spectrophotometrically. Alternatively, the enzyme may alter the fluorescence or chemiluminescence of the substrate. Techniques for quantifying a change in fluorescence are described above. The chemiluminescent substrate becomes electronically excited by a chemical reaction and may then emit light which can be measured (using a chemiluminometer, for example) or donates energy to a fluorescent acceptor. Examples of enzymatic labels include luciferases (*e.g.*, firefly luciferase and bacterial luciferase; U.S. Patent No. 4,737,456), luciferin, 2,3-dihydrophthalazinediones, malate dehydrogenase, urease, peroxidase such as horseradish peroxidase (HRPO), alkaline phosphatase, β-galactosidase, glucoamylase, lysozyme, saccharide oxidases (*e.g.*, glucose oxidase, galactose oxidase, and glucose-6-phosphate dehydrogenase), heterocyclic oxidases (such as uricase and xanthine oxidase), lactoperoxidase, microperoxidase, and the like. Techniques for conjugating enzymes to antibodies are described in O'Sullivan *et al.*, Methods for the Preparation of Enzyme-Antibody Conjugates for use in Enzyme Immunoassay, in *Methods in Enzym.* (ed J. Langone & H. Van Vunakis), Academic press, New York, 73:147-166 (1981).

Examples of enzyme-substrate combinations include, for example:

(i) Horseradish peroxidase (HRPO) with hydrogen peroxidase as a substrate, wherein the hydrogen peroxidase oxidizes a dye precursor (*e.g.*, orthophenylene diamine (OPD) or 3,3',5,5'-tetramethyl benzidine hydrochloride (TMB));

(ii) alkaline phosphatase (AP) with para-Nitrophenyl phosphate as chromogenic substrate; and

(iii) β-D-galactosidase (β-D-Gal) with a chromogenic substrate (*e.g.*, p-nitrophenyl-β-D-galactosidase) or fluorogenic substrate 4-methylumbelliferyl-β-D-galactosidase.

Numerous other enzyme-substrate combinations are available to those skilled in the art. For a general review of these, see U.S. Patent Nos. 4,275,149 and 4,318,980.

Sometimes, the label is indirectly conjugated with the antibody. The skilled artisan

will be aware of various techniques for achieving this. For example, the antibody can be conjugated with biotin and any of the three broad categories of labels mentioned above can be conjugated with avidin, or *vice versa*. Biotin binds selectively to avidin and thus, the label can be conjugated with the antibody in this indirect manner. Alternatively, to achieve indirect conjugation of the label with the antibody, the antibody is conjugated with a small hapten (*e.g.*, digoxin) and one of the different types of labels mentioned above is conjugated with an anti-hapten antibody (*e.g.*, anti-digoxin antibody). Thus, indirect conjugation of the label with the antibody can be achieved.

In another embodiment of the invention, the anti-VEGF antibody need not be labeled, and the presence thereof can be detected using a labeled antibody which binds to the VEGF antibody.

The antibodies of the present invention may be employed in any known assay method, such as competitive binding assays, direct and indirect sandwich assays, and immunoprecipitation assays. Zola, *Monoclonal Antibodies: A Manual of Techniques*, pp.147-158 (CRC Press, Inc. 1987).

Competitive binding assays rely on the ability of a labeled standard to compete with the test sample analyte for binding with a limited amount of antibody. The amount of VEGF protein in the test sample is inversely proportional to the amount of standard that becomes bound to the antibodies. To facilitate determining the amount of standard that becomes bound, the antibodies generally are insolubilized before or after the competition, so that the standard and analyte that are bound to the antibodies may conveniently be separated from the standard and analyte which remain unbound.

Sandwich assays involve the use of two antibodies, each capable of binding to a different immunogenic portion, or epitope, of the protein to be detected. In a sandwich assay, the test sample analyte is bound by a first antibody which is immobilized on a solid support, and thereafter a second antibody binds to the analyte, thus forming an insoluble three-part complex. See, *e.g.*, US Pat No. 4,376,110. The second antibody may itself be labeled with a detectable moiety (direct sandwich assays) or may be measured using an anti-immunoglobulin antibody that is labeled with a detectable moiety (indirect sandwich assay). For example, one type of sandwich assay is an ELISA assay, in which case the detectable moiety is an enzyme.

For immunohistochemistry, the tumor sample may be fresh or frozen or may be embedded in paraffin and fixed with a preservative such as formalin, for example.

The antibodies may also be used for *in vivo* diagnostic assays. Generally, the antibody is labeled with a radio nuclide (such as ^{111}In, ^{99}Tc, ^{14}C, ^{131}I, ^{125}I, ^{3}H, ^{32}P or ^{35}S) so that the tumor can be localized using immunoscintiography.

E. Diagnostic Kits

As a matter of convenience, the antibody of the present invention can be provided in a kit, *i.e.*, a packaged combination of reagents in predetermined amounts with instructions for performing the diagnostic assay. Where the antibody is labeled with an enzyme, the kit will include substrates and cofactors required by the enzyme (*e.g.*, a substrate precursor which provides the detectable chromophore or fluorophore). In addition, other additives may be included such as stabilizers, buffers (*e.g.*, a block buffer or lysis buffer) and the like. The relative amounts of the various reagents may be varied widely to provide for concentrations in solution of the reagents which substantially optimize the sensitivity of the assay. Particularly, the reagents may be provided as dry powders, usually lyophilized, including excipients which on dissolution will provide a reagent solution having the appropriate concentration.

F. Therapeutic Uses for the Antibody

For therapeutic applications, the anti-VEGF antibodies of the invention are administered to a mammal, preferably a human, in a pharmaceutically acceptable dosage form such as those discussed above, including those that may be administered to a human intravenously as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intra-cerebrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes. The antibodies also are suitably administered by intra tumoral, peritumoral, intralesional, or perilesional routes, to exert local as well as systemic therapeutic effects. The intraperitoneal route is expected to be particularly useful, for example, in the treatment of ovarian tumors.

For the prevention or treatment of disease, the appropriate dosage of antibody will depend on the type of disease to be treated, as defined above, the severity and course of the disease, whether the antibody is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the antibody, and the discretion of the attending physician. The antibody is suitably administered to the patient at one time

or over a series of treatments.

The anti-VEGF antibodies are useful in the treatment of various neoplastic and non-neoplastic diseases and disorders. Neoplasms and related conditions that are amenable to treatment include breast carcinomas, lung carcinomas, gastric carcinomas, esophageal carcinomas, colorectal carcinomas, liver carcinomas, ovarian carcinomas, thecomas, arrhenoblastomas, cervical carcinomas, endometrial carcinoma, endometrial hyperplasia, endometriosis, fibrosarcomas, choriocarcinoma, head and neck cancer, nasopharyngeal carcinoma, laryngeal carcinomas, hepatoblastoma, Kaposi's sarcoma, melanoma, skin carcinomas, hemangioma, cavernous hemangioma, hemangioblastoma, pancreas carcinomas, retinoblastoma, astrocytoma, glioblastoma, Schwannoma, oligodendroglioma, medulloblastoma, neuroblastomas, rhabdomyosarcoma, osteogenic sarcoma, leiomyosarcomas, urinary tract carcinomas, thyroid carcinomas, Wilm's tumor, renal cell carcinoma, prostate carcinoma, abnormal vascular proliferation associated with phakomatoses, edema (such as that associated with brain tumors), and Meigs' syndrome.

Non-neoplastic conditions that are amenable to treatment include rheumatoid arthritis, psoriasis, atherosclerosis, diabetic and other proliferative retinopathies including retinopathy of prematurity, retrolental fibroplasia, neovascular glaucoma, age-related macular degeneration, thyroid hyperplasias (including Grave's disease), corneal and other tissue transplantation, chronic inflammation, lung inflammation, nephrotic syndrome, preeclampsia, ascites, pericardial effusion (such as that associated with pericarditis), and pleural effusion.

Age-related macular degeneration (AMD) is a leading cause of severe visual loss in the elderly population. The exudative form of AMD is characterized by choroidal neovascularization and retinal pigment epithelial cell detachment. Because choroidal neovascularization is associated with a dramatic worsening in prognosis, the VEGF antibodys of the present invention are expected to be especially useful in reducing the severity of AMD.

Depending on the type and severity of the disease, about 1 μg/kg to about 50 mg/kg (*e.g.*, 0.1-20mg/kg) of antibody is an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. A typical daily or weekly dosage might range from about 1 μg/kg to about 20 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment is repeated until a desired suppression of disease symptoms occurs. However, other dosage regimens may be useful.

The progress of this therapy is easily monitored by conventional techniques and assays, including, for example, radiographic tumor imaging.

According to another embodiment of the invention, the effectiveness of the antibody in preventing or treating disease may be improved by administering the antibody serially or in combination with another agent that is effective for those purposes, such as tumor necrosis factor (TNF), an antibody capable of inhibiting or neutralizing the angiogenic activity of acidic or basic fibroblast growth factor (FGF) or hepatocyte growth factor (HGF), an antibody capable of inhibiting or neutralizing the coagulant activities of tissue factor, protein C, or protein S (see Esmon *et al.*, PCT Patent Publication No. WO 91/01753, published 21 February 1991), an antibody capable of binding to HER2 receptor (see Hudziak *et al.*, PCT Patent Publication No. WO 89/06692, published 27 July 1989), or one or more conventional therapeutic agents such as, for example, alkylating agents, folic acid antagonists, anti-metabolites of nucleic acid metabolism, antibiotics, pyrimidine analogs, 5-fluorouracil, cisplatin, purine nucleosides, amines, amino acids, triazol nucleosides, or corticosteroids. Such other agents may be present in the composition being administered or may be administered separately. Also, the antibody is suitably administered serially or in combination with radiological treatments, whether involving irradiation or administration of radioactive substances.

In one embodiment, vascularization of tumors is attacked in combination therapy. The antibody and one or more other anti-VEGF antagonists are administered to tumor-bearing patients at therapeutically effective doses as determined for example by observing necrosis of the tumor or its metastatic foci, if any. This therapy is continued until such time as no further beneficial effect is observed or clinical examination shows no trace of the tumor or any metastatic foci. Then TNF is administered, alone or in combination with an auxiliary agent such as alpha-, beta-, or gamma-interferon, anti-HER2 antibody, heregulin, anti-heregulin antibody, D-factor, interleukin-1 (IL-1), interleukin-2 (IL-2), granulocyte-macrophage colony stimulating factor (GM-CSF), or agents that promote microvascular coagulation in tumors, such as anti-protein C antibody, anti-protein S antibody, or C4b binding protein (see Esmon *et al.*, PCT Patent Publication No. WO 91/01753, published 21 February 1991), or heat or radiation.

Since the auxiliary agents will vary in their effectiveness it is desirable to compare their impact on the tumor by matrix screening in conventional fashion. The administration

of anti-VEGF antibody and TNF is repeated until the desired clinical effect is achieved. Alternatively, the anti-VEGF antibody is administered together with TNF and, optionally, auxiliary agent(s). In instances where solid tumors are found in the limbs or in other locations susceptible to isolation from the general circulation, the therapeutic agents described herein are administered to the isolated tumor or organ. In other embodiments, a FGF or platelet-derived growth factor (PDGF) antagonist, such as an anti-FGF or an anti-PDGF neutralizing antibody, is administered to the patient in conjunction with the anti-VEGF antibody. Treatment with anti-VEGF antibodies optimally may be suspended during periods of wound healing or desirable neovascularization.

G. Articles of Manufacture

In another embodiment of the invention, an article of manufacture containing materials useful for the treatment of the disorders described above is provided. The article of manufacture comprises a container and a label. Suitable containers include, for example, bottles, vials, syringes, and test tubes. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which is effective for treating the condition and may have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). The active agent in the composition is the anti-VEGF antibody. The label on, or associated with, the container indicates that the composition is used for treating the condition of choice. The article of manufacture may further comprise a second container comprising a pharmaceutically-acceptable buffer, such as phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, syringes, and package inserts with instructions for use.

EXAMPLE 1

This example describes the production of humanized anti-VEGF antibodies with desirable properties from a therapeutic standpoint.

MATERIALS AND METHODS

Cloning of Murine A4.6.1 MAb and Construction of Mouse-Human Chimeric Fab: The murine anti-VEGF mAb A4.6.1 has been previously described by Kim *et al.*, *Growth Factors* 7:53 (1992) and Kim *et al. Nature* 362:841 (1993). Total RNA was isolated

from hybridoma cells producing the anti-VEGF Mab A.4.6.1 using RNAsol (TEL-TEST) and reverse-transcribed to cDNA using Oligo-dT primer and the SuperScript II system (GIBCO BRL, Gaithersburg, MD). Degenerate oligonucleotide primer pools, based of the N-terminal amino acid sequences of the light and heavy chains of the antibody, were synthesized and used as forward primers. Reverse primers were based on framework 4 sequences obtained from murine light chain subgroup kV and heavy chain subgroup II (Kabat *et al. Sequences of Proteins of Immunological Interest.* 5th ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991)). After polymerase chain reaction (PCR) amplification, DNA fragments were ligated to a TA cloning vector (Invitrogen, San Diego, CA). Eight clones each of the light and heavy chains were sequenced. One clone with a consensus sequence for the light chain VL domain and one with a consensus sequence for the heavy chain VH domain were subcloned respectively into the pEMX1 vector containing the human CL and CH1 domains (Werther *et al. J. Immunol.* 157:4986-4995 (1996)), thus generating a mouse-human chimera. This chimeric F(ab) consisted of the entire murine A4.6.1 VH domain fused to a human CH1 domain at amino acid SerH113 and the entire murine A4.6.1 VL domain fused to a human CL domain at amino acid LysL107. Expression and purification of the chimeric F(ab) were identical to that of the humanized F(ab)s. The chimeric F(ab) was used as the standard in the binding assays.

Computer Graphics Models of Murine and Humanized F(ab): Sequences of the VL and VH domains (Figs.1A and 1B) were used to construct a computer graphics model of the murine A4.6.1 VL-VH domains. This model was used to determine which framework residues should be incorporated into the humanized antibody. A model of the humanized F(ab) was also constructed to verify correct selection of murine framework residues. Construction of models was performed as described previously (Carter *et al. Proc. Natl. Acad. Sci. USA* 89:4285-4289 (1992) and Eigenbrot *et al. J.Mol. Biol.* 229:969-995 (1993)).

Construction of Humanized F(ab)s: The plasmid pEMX1 used for mutagenesis and expression of F(ab)s in *E. coli* has been described previously (Werther *et al.*, *supra*). Briefly, the plasmid contains a DNA fragment encoding a consensus human k subgroup I light chain (VLkI-CL) and a consensus human subgroup III heavy chain (VHIII-CH1) and an alkaline phosphatase promoter. The use of the consensus sequences for VL and VH has been described previously (Carter *et al.*, *supra*).

To construct the first F(ab) variant of humanized A4.6.1, F(ab)-1, site-directed mutagenesis (Kunkel *et al.*, *Proc. Natl. Acad. Sci. USA* 82:488-492 (1985)) was performed on a deoxyuridine-containing template of pEMX1. The six CDRs according to Kabat *et al.*, *supra*, were changed to the murine A4.6.1 sequence . F(ab)-1 therefore consisted of a complete human framework (VL k subgroup I and VH subgroup III) with the six complete murine CDR sequences. Plasmids for all other F(ab) variants were constructed from the plasmid template of F(ab)-1. Plasmids were transformed into *E. coli* strain XL-1 Blue (Stratagene, San Diego, CA) for preparation of double- and single-stranded DNA. For each variant, DNA coding for light and heavy chains was completely sequenced using the dideoxynucleotide method (Sequenase, U.S. Biochemical Corp., Cleveland, OH). Plasmids were transformed into *E. coli* strain 16C9, a derivative of MM294, plated onto Luria broth plates containing 50 µg/ml carbenicillin, and a single colony selected for protein expression. The single colony was grown in 5 ml Luria broth-100 mg/ml carbenicillin for 5-8 h at 37°C. The 5 ml culture was added to 500 ml AP5-50 µg/ml carbenicillin and allowed to grow for 20 h in a 4 L baffled shake flask at 30°C. AP5 media consists of: 1.5 g glucose, 11.0 g Hycase SF, 0.6 g yeast extract (certified), 0.19 g MgSO4 (anhydrous), 1.07 g NH4Cl, 3.73 g KCl, 1.2 g NaCl, 120 ml 1 M triethanolamine, pH 7.4, to 1 L water and then sterile filtered through 0.1 mm Sealkeen filter. Cells were harvested by centrifugation in a 1 L centrifuge bottle at 3000x*g* and the supernatant removed. After freezing for 1 h, the pellet was resuspended in 25 ml cold 10 mM Tris-1 mM EDTA-20% sucrose, pH 8.0. 250 ml of 0.1 M benzamidine (Sigma, St. Louis, MO) was added to inhibit proteolysis. After gentle stirring on ice for 3 h, the sample was centrifuged at 40,000x*g* for 15 min. The supernatant was then applied to a protein G-Sepharose CL-4B (Pharmacia, Uppsala, Sweden) column (0.5 ml bed volume) equilibrated with 10 mM Tris-1 mM EDTA, pH 7.5. The column was washed with 10 ml of 10 mM Tris-1 mM EDTA, pH 7.5, and eluted with 3 ml 0.3 M glycine, pH 3.0, into 1.25 ml 1 M Tris, pH 8.0. The F(ab) was then buffer exchanged into PBS using a Centricon-30 (Amicon, Beverly, MA) and concentrated to a final volume of 0.5 ml. SDS-PAGE gels of all F(ab)s were run to ascertain purity and the molecular weight of each variant was verified by electrospray mass spectrometry.

Construction and Expression of Chimeric and Humanized IgG: For generation of human IgG1 variants of chimeric (chIgG1) and humanized (huIgG1) A4.6.1, the appropriate murine or humanized VL and VH (F(ab)-12, Table 2) domains were subcloned

into separate, previously described, pRK vectors (Eaton *et al.*, *Biochemistry* 25:8343-8347 (1986)). The DNA coding for the entire light and the entire heavy chain of each variant was verified by dideoxynucleotide sequencing.

For transient expression of variants, heavy and light chain plasmids were co-transfected into human 293 cells (Graham *et al.*, *J. Gen. Virol.* 36:59-74 (1977)), using a high efficiency procedure (Gorman *et al.*, *DNA Prot. Eng. Tech.* 2:3-10 (1990)). Media was changed to serum-free and harvested daily for up to five days. Antibodies were purified from the pooled supernatants using protein A-Sepharose CL-4B (Pharmacia). The eluted antibody was buffer exchanged into PBS using a Centricon-30 (Amicon), concentrated to 0.5 ml, sterile filtered using a Millex-GV (Millipore, Bedford, MA) and stored at 4°C.

For stable expression of the final humanized IgG1 variant (rhuMAb VEGF), Chinese hamster ovary (CHO) cells were transfected with dicistronic vectors designed to coexpress both heavy and light chains (Lucas *et al.*, *Nucleic Acid Res.* 24:1774-79 (1996)). Plasmids were introduced into DP12 cells, a proprietary derivative of the CHO-K1 DUX B11 cell line developed by L. Chasin (Columbia University), via lipofection and selected for growth in GHT-free medium (Chisholm, V. High efficiency gene transfer in mammalian cells. In: Glover,DM, Hames, BD. *DNA Cloning 4. Mammalian systems.* Oxford Univ. Press, Oxford pp 1-41 (1996)). Approximately 20 unamplified clones were randomly chosen and reseeded into 96 well plates. Relative specific productivity of each colony was monitored using an ELISA to quantitate the full length human IgG accumulated in each well after 3 days and a fluorescent dye, Calcien AM, as a surrogate marker of viable cell number per well. Based on these data, several unamplified clones were chosen for further amplification in the presence of increasing concentrations of methotrexate. Individual clones surviving at 10, 50, and 100 nM methotrexate were chosen and transferred to 96 well plates for productivity screening. One clone, which reproducibly exhibited high specific productivity, was expanded in T-flasks and used to inoculate a spinner culture. After several passages, the suspension-adapted cells were used to inoculate production cultures in GHT-containing, serum-free media supplemented with various hormones and protein hydrolysates. Harvested cell culture fluid containing rhuMAb VEGF was purified using protein A-Sepharose CL-4B. The purity after this step was ~99% . Subsequent purification to homogeneity was carried out using an ion exchange chromatography step. The endotoxin content of the final purified antibody was < 0.10 eu/mg.

F(ab) and IgG Quantitation: For quantitating F(ab) molecules, ELISA plates were coated with 2 µg/ml goat anti-human IgG Fab (Organon Teknika, Durham, NC) in 50 mM carbonate buffer, pH 9.6, at 4°C overnight and blocked with PBS-0.5% bovine serum albumin (blocking buffer) at room temperature for 1 h. Standards (0.78 - 50 ng/ml human F(ab)) were purchased from Chemicon (Temecula, CA). Serial dilutions of samples in PBS-0.5% bovine serum albumin-0.05% polysorbate 20 (assay buffer) were incubated on the plates for 2 h. Bound F(ab) was detected using horseradish peroxidase-labeled goat anti-human IgG F(ab) (Organon Teknika), followed by 3,3',5,5'-tetramethylbenzidine (Kirkegaard & Perry Laboratories, Gaithersburg, MD) as the substrate. Plates were washed between steps. Absorbance was read at 450 nm on a Vmax plate reader (Molecular Devices, Menlo Park, CA). The standard curve was fit using a four-parameter nonlinear regression curve-fitting program. Data points which fell in the range of the standard curve were used for calculating the F(ab) concentrations of samples. The concentration of full-length antibody was determined using goat anti-human IgG Fc (Cappel, Westchester, PA) for capture and horseradish peroxidase-labeled goat anti-human Fc (Cappel) for detection. Human IgG1 (Chemicon) was used as standard.

VEGF Binding Assay: For measuring the VEGF binding activity of F(ab)s, ELISA plates were coated with 2 µg/ml rabbit $F(ab')_2$ to human IgG Fc (Jackson ImmunoResearch, West Grove, PA) and blocked with blocking buffer (described above). Diluted conditioned medium containing 3 ng/ml of KDR-IgG (Park *et al., J. Biol. Chem.* 269:25646-25645 (1994)) in blocking buffer were incubated on the plate for 1 h. Standards (6.9 - 440 ng/ml chimeric F(ab)) and two-fold serial of samples were incubated with 2 nM biotinylated VEGF for 1 h in tubes. The solutions from the tubes were then transferred to the ELISA plates and incubated for 1 h. After washing, biotinylated VEGF bound to KDR was detected using horseradish peroxidase-labeled streptavidin (Zymed, South San Francisco, CA or Sigma, St. Louis, MO) followed by 3,3',5,5'-tetramethylbenzidine as the substrate. Titration curves were fit with a four-parameter nonlinear regression curve-fitting program (KaleidaGraph, Synergy Software, Reading PA). Concentrations of F(ab) variants corresponding to the midpoint absorbance of the titration curve of the standard were calculated and then divided by the concentration of the standard corresponding to the midpoint absorbance of the standard titration curve. Assays for full-length IgG were the same as for the F(ab)s except that the assay buffer contained 10% human serum.

BIAcore™ Biosensor Assay: VEGF binding of the humanized and chimeric F(ab)s were compared using a BIAcore™ biosensor (Karlsson *et al. Methods: A Comparison to Methods in Enzymology* 6:97-108 (1994)). Concentrations of F(ab)s were determined by quantitative amino acid analysis. VEGF was coupled to a CM-5 biosensor chip through primary amine groups according to manufacturer's instructions (Pharmacia). Off-rate kinetics were measured by saturating the chip with F(ab) (35 µl of 2 µM F(ab) at a flow rate of 20 µl/min) and then switching to buffer (PBS-0.05% polysorbate 20). Data points from 0 - 4500 sec were used for off-rate kinetic analysis. The dissociation rate constant (k_{off}) was obtained from the slope of the plot of ln(R0/R) versus time, where R0 is the signal at t=0 and R is the signal at each time point.

On-rate kinetics were measured using two-fold serial dilutions of F(ab) (0.0625 - 2 mM). The slope, K_s, was obtained from the plot of ln(-dR/dt) versus time for each F(ab) concentration using the BIAcore™ kinetics evaluation software as described in the Pharmacia Biosensor manual. R is the signal at time t. Data between 80 and 168, 148, 128, 114, 102, and 92 sec were used for 0.0625, 0.125, 0.25, 0.5, 1, and 2 mM F(ab), respectively. The association rate constant (k_{on}) was obtained from the slope of the plot of K_s versus F(ab) concentration. At the end of each cycle, bound F(ab) was removed by injecting 5 µl of 50 mM HCl at a flow rate of 20 µl/min to regenerate the chip.

Endothelial Cell Growth Assay: Bovine adrenal cortex-derived capillary endothelial cells were cultured in the presence of low glucose Dulbecco's modified Eagle's medium (DMEM) (GIBCO) supplemented with 10% calf serum, 2 mM glutamine, and antibiotics (growth medium), essentially as previously described (Leung *et al. Science* 246:1306-1309 (1989)). For mitogenic assays, endothelial cells were seeded at a density of 6×10^3 cells per well, in 6-well plates in growth medium. Either muMAb VEGF A.4.6.1 or rhuMAb VEGF was then added at concentrations ranging between 1 and 5000 ng/ml. After 2-3 hr, purified *E.coli*-expressed rhVEGF165 was added to a final concentration of 3 ng/ml. For specificity control, each antibody was added to endothelial cells at the concentration of 5000 ng/ml, either alone or in the presence of 2 ng/ml bFGF. After five or six days, cells were dissociated by exposure to trypsin and counted in a Coulter counter (Coulter Electronics, Hialeah, FL). The variation from the mean did not exceed 10%. Data were analyzed by a four-parameter curve fitting program (KaleidaGraph).

In Vivo Tumor Studies: Human A673 rhabdomyosarcoma cells (ATCC; CRL 1598) were cultured as previously described in DMEM/F12 supplemented with 10% fetal bovine serum, 2 mM glutamine and antibiotics (Kim *et al. Nature* 362:841-844 (1993) and Borgström *et al. Cancer Res.* 56:4032-4039 (1996)). Female BALB/c nude mice, 6-10 weeks old, were injected subcutaneously with 2 x 10^6 tumor cells in the dorsal area in a volume of 200 µl. Animals were then treated with muMAb VEGF A.4.6.1, rhuMAb VEGF or a control MAb directed against the gp120 protein (Kim *et al. Nature* 362:841-844 (1993)). Both anti-VEGF MAbs were administered at the doses of 0.5 and 5 mg/kg; the control MAb was given at the dose of 5 mg/kg. Each MAb was administered twice weekly intra peritoneally in a volume of 100 µl, starting 24 hr after tumor cell inoculation. Each group consisted of 10 mice. Tumor size was determined at weekly intervals. Four weeks after tumor cell inoculation, animals were euthanized and the tumors were removed and weighed. Statistical analysis was performed by ANOVA.

RESULTS

Humanization: The consensus sequence for the human heavy chain subgroup III and the light chain subgroup k I were used as the framework for the humanization (Kabat *et al.*, *supra*) (Figs. 1A and 1B). This framework has been successfully used in the humanization of other murine antibodies (Werther *et al.*, *supra*; Carter *et al.*, *supra*; Presta *et al. J. Immunol.* 151:2623-2632 (1993); and Eigenbrot *et al. Proteins* 18:49-62 (1994)). CDR-H1 included residues H26-H35. The other CDRs were according to Kabat *et al.*, *supra*. All humanized variants were initially made and screened for binding as F(ab)s expressed in *E. coli*. Typical yields from 500 ml shake flasks were 0.1-0.4 mg F(ab).

The chimeric F(ab) was used as the standard in the binding assays. In the initial variant, F(ab)-1, the CDR residues were transferred from the murine antibody to the human framework and, based on the models of the murine and humanized F(ab)s, the residue at position H49 (Ala in human) was changed to the murine Gly. In addition, F(ab)s which consisted of the chimeric heavy chain/F(ab)-1 light chain (F(ab)-2) and F(ab)-1 heavy chain/chimeric light chain (F(ab)-3) were generated and tested for binding. F(ab)-1 exhibited a binding affinity greater than 1000-fold reduced from the chimeric F(ab) (Table 2). Comparing the binding affinities of F(ab)-2 and F(ab)-3 suggested that framework residues in the F(ab)-1 VH domain needed to be altered in order to increase binding.

Table 2: Binding of Humanized Anti-VEGF F(ab) Variants to VEGF[a]

Variant	Template	Changes[b]	Purpose	EC50 F(ab)-X / EC50 chimeric F(ab)[c]		
				Mean	S.D.	N
chim-F(ab)	Chimeric F(ab)			1.0		
F(ab)-1	Human FR		Straight CDR swap AlaH49Gly	>1350		2
F(ab)-2			Chimera Light Chain F(ab)-1 Heavy Chain	>145		3
F(ab)-3			F(ab)-1 Light Chain Chimera Heavy Chain	2.6	0.1	2
F(ab)-4	F(ab)-1	ArgH71Leu AsnH73Thr	CDR-H2 conformation Framework	>295		3
F(ab)-5	F(ab)-4	LeuL46Val	VL-VH interface	80.9	6.5	2
F(ab)-6	F(ab)-5	LeuH78Ala	CDR-H1 conformation	36.4	4.2	2
F(ab)-7	F(ab)-5	IleH69Phe	CDR-H2 conformation	45.2	2.3	2
F(ab)-8	F(ab)-5	IleH69Phe LeuH78Ala	CDR-H2 conformation CDR-H1 conformation	9.6	0.9	4
F(ab)-9	F(ab)-8	GlyH49Ala	CDR-H2 conformation	>150		2
F(ab)-10	F(ab)-8	AsnH76Ser	Framework	6.4	1.2	4
F(ab)-11	F(ab)-10	LysH75Ala	Framework	3.3	0.4	2
F(ab)-12	F(ab)-10	ArgH94Lys	CDR-H3 conformation	1.6	0.6	4

[a]Anti-VEGF F(ab) variants were incubated with biotinylated VEGF and then transferred to ELISA plates coated with KDR-IgG (Park *et al.*, *supra*).
[b]Murine residues are underlined; residue numbers are according to Kabat *et al.*, *supra*.
[c]Mean and standard deviation are the average of the ratios calculated for each of the independent assays; the EC50 for chimeric F(ab) was 0.049 ± 0.013 mg/ml (1.0 nM).

Changing human residues H71 and H73 to their murine counterparts in F(ab)-4 improved binding by 4-fold (Table 2). Inspection of the models of the murine and humanized F(ab)s suggested that residue L46, buried at the VL-VH interface and interacting with CDR-H3 (Fig. 2), might also play a role either in determining the conformation of CDR-H3 and/or

affecting the relationship of the VL and VH domains. When the murine Val was exchanged for the human Leu at L46 (F(ab)-5), the binding affinity increased by almost 4-fold (Table 2). Three other buried framework residues were evaluated based on the molecular models: H49, H69 and H78. Position H69 may affect the conformation of CDR-H2 while position H78 may affect the conformation of CDR-H1 (Figure 2). When each was individually changed from the human to murine counterpart, the binding improved by 2-fold in each case (F(ab)-6 and F(ab)-7, Table 2). When both were simultaneously changed, the improvement in binding was 8-fold (F(ab)-8, Table 2). Residue H49 was originally included as the murine Gly; when changed to the human consensus counterpart Ala the binding was reduced by 15-fold (F(ab)-9, Table 2).

In F(ab)-10 and F(ab)-11 two residues in framework loop 3, FR-3, were changed to their murine counterparts: AsnH76 to murine Ser (F(ab)-10) and LysH75 to murine Ala (F(ab)-11). Both effected a relatively small improvement in binding (Table 2). Finally, at position H94 human and murine sequences most often have an Arg (Kabat *et al.*, *supra*). In F(ab)-12, this Arg was replaced by the rare Lys found in the murine antibody (Fig.1A) and this resulted in binding which was less than 2-fold from the chimeric F(ab) (Table 2). F(ab)-12 was also compared to the chimeric F(ab) using the BIAcore™ system (Pharmacia). Using this technique the K_d of the humanized F(ab)-12 was 2-fold weaker than that of the chimeric F(ab) due to both a slower k_{on} and faster k_{off} (Table 3).

Table 3: Binding of Anti-VEGF F(ab) Variants to VEGF Using the BIAcore™ System[a]

Variant	Amount of (Fab) bound (RU)	k_{off} (s^{-1})	k_{on} ($M^{-1}s^{-1}$)	K_d (nM)
chim-F(ab)[b]	4250	$5.9x10^{-5}$	$6.5x10^{4}$	0.91
F(ab)-12	3740	$6.3x10^{-5}$	$3.5x10^{4}$	1.8

[a] The amount of F(ab) bound, in resonance units (RU), was measured using a BIAcore™ system when 2 µg F(ab) was injected onto a chip containing 2480 RU immobilized VEGF. Off-rate kinetics (k_{off}) were measured by saturating the chip with F(ab) and then monitoring dissociation after switching to buffer. On-rate kinetics (k_{on}) were measured using two-fold serial dilutions of F(ab). K_d, the equilibrium dissociation constant, was calculated as k_{off}/k_{on}.
[b] chim-F(ab) is a chimeric F(ab) with murine VL and VH domains fused to human CL and CH1 heavy domains.

Full length mAbs were constructed by fusing the VL and VH domains of the chimeric F(ab) and variant F(ab)-12 to the constant domains of human k light chain and human IgG1 heavy chain. The full length 12-IgG1 (F(ab)-12 fused to human IgG1) exhibited binding which was 1.7-fold weaker than the chimeric IgG1 (Table 4). Both 12-IgG1 and the chimeric IgG1 bound slightly less well than the original murine mAb A4.6.1 (Table 4).

Table 4: Binding of Anti-VEGF IgG Variants to VEGF[a]

IgG1/chIgG1[b]			
Variant	**Mean**	**S.D.**	**N**
chIgG1	1.0		2
murIgG1[c]	0.759	0.001	2
12-IgG1[d]	1.71	0.03	2

[a]Anti-VEGF IgG variants were incubated with biotinylated VEGF and then transferred to ELISA plates coated with KDR-IgG (Park *et al.*, (1994), *supra*).
[b]chIgG1 is chimeric IgG1 with murine VL and VH domains fused to human CL and IgG1 heavy chains; the EC50 for chIgG1 was 0.113 ± 0.013 µg/ml (0.75 nM).
[c]murIgG1 is muMAbVEGF A461 purified from ascites.
[d]12-IgG1 is F(ab)-12 VL and VH domains fused to human CL and IgG1 heavy chains.

Biological Studies: rhuMAb VEGF and muMAb VEGF A.4.6.1. were compared for their ability to inhibit bovine capillary endothelial cell proliferation in response to a near maximally effective concentration of VEGF (3 ng/ml). As illustrated in Figure 3, the two MAbs were essentially equivalent, both in potency and efficacy. The ED50 values were respectively 50 ± 5 ng/ml and 48 ± 8 ng/ml (~0.3 nM). In both cases 90% inhibition was achieved at the concentration of 500 ng/ml (~3 nM). Neither muMAb VEGF A.4.6.1 nor rhuMAb VEGF had any effect on basal or bFGF-stimulated proliferation of capillary endothelial cells, confirming that the inhibition is specific for VEGF.

To determine whether such equivalency applies also to an *in vivo* system, the two antibodies were compared for their ability to suppress the growth of human A673 rhabdomyosarcoma cells in nude mice. Previous studies have shown that muMAb VEGF A.4.6.1 has a dramatic inhibitory effect in this tumor model (Kim *et al. Nature* 362:841-844

(1993) and Borgström *et al. Cancer Res* 56:4032-4039 (1996)). As shown in Figure 4, at both doses tested (0.5 and 5 mg/kg), the two antibodies markedly suppressed tumor growth as assessed by tumor weight measurements four weeks after cell inoculation. The decreases in tumor weight compared to the control group were respectively 85% and 93% at each dose in the animals treated with muMAb VEGF A.4.6.1. versus 90% and 95% in those treated with rhuMAb VEGF. Similar results were obtained with the breast carcinoma cell line MDA-MB 435.

EXAMPLE 2

In this example, the murine anti-VEGF antibody A4.6.1 discussed above was humanized by randomizing a small set of framework residues and by monovalent display of the resultant library of antibody molecules on the surface of filamentous phage in order to identify high affinity framework sequences via affinity-based selection.

MATERIALS AND METHODS

Construction of Anti-VEGF Phagemid Vector, pMB4-19: The murine anti-VEGF mAb A4.6.1 is discussed above in Example 1. The first Fab variant of humanized A4.6.1, hu2.0, was constructed by site-directed mutagenesis using a deoxyuridine-containing template of plasmid pAK2 (Carter *et al. Proc. Natl. Acad. Sci. U.S.A.* 89:4285-4289 (1992)) which codes for a human $V_L\kappa$I-Cκ_1 light chain and human V_HIII-$C_H1\gamma_1$ heavy chain Fd fragment The transplanted A4.6.1 CDR sequences were chosen according to the sequence definition of Kabat *et al.*, *supra*, except for CDR-H1 which included residues 26-35. The Fab encoding sequence was subcloned into the phagemid vector phGHamg3 (Bass *et al. Proteins* 8:309-314 (1990) and Lowman *et al. Biochemistry* 30:10832-10838 (1991)). This construct, pMB4-19, encodes the initial humanized A4.6.1 Fab, hu2.0, with the C-terminus of the heavy chain fused precisely to the carboxyl portion of the M13 gene III coat protein. pMB4-19 is similar in construction to pDH188, a previously described plasmid for monovalent display of Fab fragments (Garrard *et al. Biotechnology* 9:1373-1377 (1991)). Notable differences between pMB4-19 and pDH188 include a shorter M13 gene III segment (codons 249-406) and use of an amber stop codon immediately following the antibody heavy chain Fd fragment. This permits expression of both secreted heavy chain or heavy chain-gene III fusions in *supE* suppressor strains of *E. coli*.

Expression and Purification of Humanized A4.6.1 Fab Fragment: *E. coli* strain 34B8, a nonsuppressor, was transformed with phagemid pMB4-19, or variants thereof.

Single colonies were grown overnight at 37°C in 5 mL 2YT containing 50 µg/mL carbenicillin. These cultures were diluted into 200 mL AP5 medium (Chang *et al. Gene* 55:189-196 (1987)) containing 20 µg/mL carbenicillin and incubated for 26 hr at 30°C. The cells were pelleted at 4000 x g and frozen at -20°C for at least 2 h. Cell pellets were then resuspended in 5 mL of 10 mM Tris-HCl (pH 7.6) containing 1 mM EDTA, shaken at 4°C for 90 min and centrifuged at 10,000 x g for 15 min. The supernatant was applied to a 1 mL streptococcal protein G-sepharose column (Pharmacia) and washed with 10 mL of 10 mM MES (pH 5.5). The bound Fab fragment was eluted with 2.5 mL 100 mM acetic acid and immediately neutralized with 0.75 mL 1M Tris-HCl, pH 8.0. Fab preparations were buffer-exchanged into PBS and concentrated using Centricon-30 concentrators (Amicon). Typical yields of Fab were ~1 mg/L culture, post-protein G purification. Purified Fab samples were characterized by electrospray mass spectrometry, and concentrations were determined by amino acid analysis.

Construction of the Anti-VEGF Fab Phagemid Library: The humanized A4.6.1 phagemid library was constructed by site-directed mutagenesis according to the method of Kunkel *et al. Methods Enzymol.* 204:125-139 (1991)). A derivative of pMB4-19 containing TAA stop triplets at V_H codons 24, 37, 67 and 93 was prepared for use as the mutagenesis template (all sequence numbering according to Kabat *et al.*, *supra*). This modification was to prevent subsequent background contamination by wild type sequences. The codons targeted for randomization were 4 and 71 (light chain) and 24, 37, 67, 69, 71, 73, 75, 76, 78, 93 and 94 (heavy chain).

In order to randomize heavy chain codons 67, 69, 71, 73, 75, 76, 78, 93 and 94 with a single mutagenic oligonucleotide, two 126-mer oligonucleotides were first preassembled from 60 and 66-mer fragments by template-assisted enzymatic ligation. Specifically, 1.5 nmol of 5' phosphorylated oligonucleotide 503-1 (5'-GAT TTC AAA CGT CGT NYT ACT WTT TCT AGA GAC AAC TCC AAA AAC ACA BYT TAC CTG CAG ATG AAC-3' (SEQ ID NO:22)) or 503-2 (5'-GAT TTC AAA CGT CGT NYT ACT WTT TCT TTA GAC ACC TCC GCA AGC ACA BYT TAC CTG CAG ATG AAC-3' (SEQ ID NO:23)) were combined with 1.5 nmol of 503-3 (5'-AGC CTG CGC GCT GAG GAC ACT GCC GTC TAT TAC TGT DYA ARG TAC CCC CAC TAT TAT GGG-3' (SEQ ID NO:24)) (randomized codons underlined; N=A/G/T/C; W=A/T; B=G/T/C; D=G/A/T; R=A/G; Y=C/T). Then, 1.5 nmol of template oligonucleotide (5'-CTC AGC GCG CAG GCT GTT

CAT CTG CAG GTA-3' (SEQ ID NO:25)), with complementary sequence to the 5' ends of 503-1/2 and the 3' end of 503-3, was added to hybridize to each end of the ligation junction. *Taq* ligase (thermostable ligase from New England Biolabs) and buffer were added, and the reaction mixture was subjected to 40 rounds of thermal cycling, (95°C 1.25 min; 50°C for 5 min) so as to cycle the template oligonucleotide between ligated and unligated junctions. The product 126-mer oligonucleotides were purified on a 6% urea/TBE polyacrylamide gel and extracted from the polyacrylamide in buffer. The two 126-mer products were combined in equal ratio, ethanol precipitated and finally solubilized in 10mM Tris-HCl, 1mM EDTA. The mixed 126-mer oligonucleotide product was labeled 504-01.

Randomization of select framework codons (V_L 4, 71; V_H 24, 37, 67, 69, 71, 73, 75, 76, 93, 94) was effected in two steps. Firstly, V_L randomization was achieved by preparing three additional derivatives of the modified pMB4-19 template. Framework codons 4 and 71 in the light chain were replaced individually or pairwise using the two mutagenic oligonucleotides 5'-GCT GAT ATC CAG TTG ACC CAG TCC CCG-3' (SEQ ID NO:26) 5'-and TCT GGG ACG GAT TAC ACT CTG ACC ATC-3' (SEQ ID NO:27). Deoxyuridine-containing template was prepared from each of these new derivatives. Together with the original template, these four constructs coded for each of the four possible light chain framework sequence combinations (Table 5).

Oligonucleotides 504-1, a mixture of two 126-mer oligonucleotides (see above), and 5'-CGT TTG TCC TGT GCA RYT TCT GGC TAT ACC TTC ACC AAC TAT GGT ATG AAC TGG RTC CGT CAG GCC CCG GGT AAG-3' (SEQ ID NO:28) were used to randomize heavy chain framework codons using each of the four templates just described. The four libraries were electroporated into *E. coli* XL-1 Blue cells (Stratagene) and combined. The total number of independent transformants was estimated at $>1.2 \times 10^8$, approximately 1,500-fold greater than the maximum number of DNA sequences in the library.

A variety of systems have been developed for the functional display of antibody fragments on the surface of filamentous phage. Winter *et al.*, *Ann. Rev. Immunol.* 12,433 (1994). These include the display of Fab or single chain Fv (scFv) fragments as fusions to either the gene III or gene VIII coat proteins of M13 bacteriophage. The system selected herein is similar to that described by Garrard *et al.*, *Biotechn*, 9,1373 (1991) in which a Fab fragment is monovalently displayed as a gene III fusion (Figure 7). This system has two notable features. In particular, unlike scFvs, Fab fragments have no tendency to form dimeric

species, the presence of which can prevent selection of the tightest binders due to avidity effects. Additionally the monovalency of the displayed protein eliminates a second potential source of avidity effects that would otherwise result from the presence of multiple copies of a protein on each phagemid particle. Bass and Wells, *Proteins* 8:309 (1990) and Lowman *et al.*, *Biochemistry* 30:10832 (1991).

Phagemid particles displaying the humanized A4.6.1 Fab fragments were propagated in *E. coli* XL-1 Blue cells. Briefly, cells harboring the randomized pMB4-19 construct were grown overnight at 37°C in 25 mL 2YT medium containing 50μg/mL carbenicillin and approximately 10^{10} M13KO7 helper phage (Vieira & Messing *Methods Enzymol.* 153:3-11 (1987)). Phagemid stocks were purified from culture supernatants by precipitation with a saline polyethylene glycol solution, and resuspended in 100 μL PBS (~10^{14} phagemid/mL)

Selection of Humanized A4.6.1 Fab Variants: Purified $VEGF_{121}$ (100 μL at 10μg/mL in PBS) was coated onto a microtiter plate well overnight at 4°C. The coating solution was discarded and this well, in addition to an uncoated well, were blocked with 6% skim milk for 1 h and washed with PBS containing 0.05% TWEEN 20™ (detergent). Then, 10 μL of phagemid stock, diluted to 100 μL with 20 mM Tris (pH 7.5) containing 0.1% BSA and 0.05%TWEEN 20™, was added to each well. After 2 hours the wells were washed and the bound phage eluted with 100 μL of 0.1 M glycine (pH 2.0), and neutralized with 25 μL of 1M Tris pH 8.0. An aliquot of this was used to titer the number of phage eluted. The remaining phage eluted from the VEGF-coated well were propagated for use in the next selection cycle. A total of 8 rounds of selection was performed after which time 20 individual clones were selected and sequenced (Sanger *et al. Proc. Natl. Acad. Sci. U.S.A.* 74:5463-5467 (1977)).

Determination of VEGF Binding Affinities: Association (k_{on}) and dissociation (k_{off}) rate constants for binding of humanized A4.6.1 Fab variants to $VEGF_{121}$ were measured by surface plasmon resonance (Karlsson *et al. J. Immun. Methods* 145:229-240 (1991)) on a Pharmacia BIAcore instrument. $VEGF_{121}$ was covalently immobilized on the biosensor chip via primary amino groups. Binding of humanized A4.6.1 Fab variants was measured by flowing solutions of Fab in PBS/0.05% TWEEN 20™ over the chip at a flow rate of 20 μL/min. Following each binding measurement, residual Fab was stripped from the immobilized ligand by washing with 5 μL of 50 mM aqueous HCl at 3 μL/min. Binding

profiles were analyzed by nonlinear regression using a simple monovalent binding model (BIAevaluation software v2.0; Pharmacia).

RESULTS

Construction of Humanized A4.6.1: An initial humanized A4.6.1 Fab fragment was constructed (hu2.0, Figs. 5A and 5B), in which the CDRs from A4.6.1 were grafted onto a human $V_L\kappa I$-$V_H III$ framework. All other residues in hu2.0 were maintained as the human sequence. Binding of this variant to VEGF was so weak as to be undetectable. Based on the relative affinity of other weakly-binding humanized A4.6.1 variants, the K_D for binding of hu2.0 was estimated at >7 μM. This contrasts with an affinity of 1.6 nM for a chimeric Fab construct consisting of the intact V_L and V_H domains from murine A4.6.1 and human constant domains. Thus binding of hu2.0 to VEGF was at least 4000-fold reduced relative to the chimera.

Design of Antibody Library: The group of framework changes to the human framework sequence herein is shown in Table 5 and Fig. 6.

Table 5: Key Framework Residues Important for Antigen Binding and Targeted for Randomization

Framework residue		Human $V\kappa_L I$, $V_H III$ consensus residue	Murine A4.6.1 residue	Randomization[a]
V_L:	4	Met	Met	Met, Leu
	71	Phe	Tyr	Phe, Tyr
V_H:	24	Ala	Ala	Ala, Val, Thr
	37	Val	Val	Val, Ile
	67	Phe	Phe	Phe, Val, Thr, Leu, Ile, Ala
	69	Ile	Phe	Ile, Phe
	71	Arg	Leu	Arg[b], Leu[b]
	73	Asp	Thr	Asp[b], Thr[b]
	75	Lys	Ala	Lys[b], Ala[b]
	76	Asn	Ser	Asn[b], Ser[b]

Framework residue		Human $V\kappa_L I$, $V_H III$ consensus residue	Murine A4.6.1 residue	Randomization[a]
	78	Leu	Ala	Leu, Ala, Val, Phe
	93	Ala	Ala	Ala, Val, Leu, Ser, Thr
	94	Arg	Lys	Arg, Lys

[a]Amino acid diversity in phagemid library

[b]V_H71, 73, 75, 76 randomized to yield the all-murine (L71/T73/A75/S76) or all-human (R71/D73/K75/N76) V_HIII tetrad

A concern in designing the humanized A4.6.1 phagemid library was that residues targeted for randomization were widely distributed across the V_L and V_H sequences. Limitations in the length of synthetic oligonucleotides requires that simultaneous randomization of each of these framework positions can only be achieved through the use of multiple oligonucleotides. However, as the total number of oligonucleotides increases, the efficiency of mutagenesis decreases (*i.e.* the proportion of mutants obtained which incorporate sequence derived from all of the mutagenic oligonucleotides). To circumvent this problem, two features were incorporated into the library construction. The first was to prepare four different mutagenesis templates coding for each of the possible V_L framework combinations. This was simple to do given the limited diversity of the light chain framework (only 4 different sequences), but was beneficial in that it eliminated the need for two oligonucleotides from the mutagenesis strategy. Secondly, two 126-base oligonucleotides were preassembled from smaller synthetic fragments. This made possible randomization of V_H codons 67, 69, 71, 73, 75, 76, 93 and 94 with a single long oligonucleotide, rather than two smaller ones. The final randomization mutagenesis strategy therefore employed only two oligonucleotides simultaneously onto four different templates.

Selection of Tight Binding Humanized A4.6.1 Fab's: Variants from the humanized A4.6.1 Fab phagemid library were selected based on binding to VEGF. Enrichment of functional phagemid, as measured by comparing titers for phage eluted from a VEGF-coated versus uncoated microtiter plate well, increased up to the seventh round of affinity panning. After one additional round of sorting, 20 clones were sequenced to identify preferred framework residues selected at each position randomized. These results, summarized in Table 6, revealed strong consensus amongst the clones selected. Ten out of

the twenty clones had the identical DNA sequence, designated hu2.10. Of the thirteen framework positions randomized, eight substitutions were selected in hu2.10 (V_L 71; V_H 37, 71, 73, 75, 76, 78 and 94). Interestingly, residues V_H 37 (Ile) and 78 (Val) were selected neither as the human V_HIII or murine A4.6.1 sequence. This result suggests that some framework positions may benefit from extending the diversity beyond the target human and parent murine framework sequences.

Table 6: Sequences Selected from the Humanized A4.6.1 Phagemid Fab Library

Variant	**Residue substitutions**												
	V_L		V_H										
	4	71	24	37	67	69	71	73	75	76	78	93	94
murine A4.6.1	M	Y	A	V	F	F	L	T	A	S	A	A	K
hu2.0 (CDR-graft)	M	F	A	V	F	I	R	N	K	N	L	A	R
Phage-selected clones:													
hu2.1(2)	-	Y	-	I	-	-	-	-	-	-	V	-	K
hu2.2(2)	L	Y	-	I	-	-	-	-	-	-	V	-	K
hu2.6(1)	L	-	-	I	T	-	L	T	A	S	V	-	K
hu2.7(1)	L	-	-	I	-	-	-	-	-	-	V	-	K
hu2.10(10)	-	Y	-	I	-	-	L	T	A	S	V	-	K

Differences between hu2.0 and murine A4.6.1 antibodies are underlined. The number of identical clones identifies for each phage-selected sequence is indicated in parentheses. Dashes in the sequences of phage-selected clones indicate selection of the human V_LKI-V_HIII framework sequence (*i.e.* as in hu2.0).

There were four other unique amino acid sequences among the remaining ten clones analyzed: hu2.1, hu2.2, hu2.6 and hu2.7. All of these clones, in addition to hu2.10, contained identical framework substitutions at positions V_H 37 (Ile), 78 (Val) and 94 (Lys), but retained the human V_HIII consensus sequence at positions 24 and 93. Four clones had lost the light chain coding sequence and did not bind VEGF when tested in a phage ELISA

assay (Cunningham *et al. EMBO J.* 13:2508-251 (1994)). Such artifacts can often be minimized by reducing the number of sorting cycles or by propagating libraries on solid media.

Expression and Binding Affinity of Humanized A4.6.1 Variants: Phage-selected variants hu2.1, hu2.2, hu2.6, hu2.7 and hu2.10 were expressed in *E. coli* using shake flasks and Fab fragments were purified from periplasmic extracts by protein G affinity chromatography. Recovered yields of Fab for these five clones ranged from 0.2 (hu2.6) to 1.7 mg/L (hu2.1). The affinity of each of these variants for antigen (VEGF) was measured by surface plasmon resonance on a BIAcore instrument (Table 7). Analysis of this binding data revealed that the consensus clone hu2.10 possessed the highest affinity for VEGF out of the five variants tested. Thus the Fab phagemid library was selectively enriched for the tightest binding clone. The calculated K_D for hu2.10 was 55 nM, at least 125-fold tighter than for hu2.0 which contains no framework changes (K_D >7 μM). The other four selected variants all exhibited weaker binding to VEGF, ranging down to a K_D of 360 nM for the weakest (hu2.7). Interestingly, the K_D for hu2.6, 67 nM, was only marginally weaker than that of hu2.10 and yet only one copy of this clone was found among 20 clones sequenced. This may have due to a lower level of expression and display, as was the case when expressing the soluble Fab of this variant. However, despite the lower expression rate, this variant is useful as a humanized antibody.

Table 7: VEGF Binding Affinity of Humanized A4.6.1 Fab Variants

Variant	k_{on} $M^{-1}s^{-1}/10^4$	k_{off} 10^4s^{-1}	K_D nM	$\frac{K_D(A4.6.1)}{K_D(mut)}$
A4.6.1 chimera	5.4	0.85	1.6	›4000
hu2.0	ND	ND	›7000**	
Phage selected clones:				
hu2.1	0.70	18	260	170
hu2.2	0.47	16	340	210
hu2.6	0.67	4.5	67	40
hu2.7	0.67	24	360	230
hu2.10	0.63	3.5	55	35
*hu2.10V	2.0	1.8	9.3	5.8

*hu2.10V = hu2.10 with mutation V_L Leu->Val
Estimated errors in the Biacore binding measurements are +/-25%.
**Too weak to measure; estimate of lower bound

Additional Improvement of Humanized Variant hu2.1: Despite the large improvement in antigen affinity over the initial humanized variant, binding of hu2.10 to VEGF was still 35-fold weaker than a chimeric Fab fragment containing the murine A4.6.1 V_L and V_H domains. This considerable difference suggested that further optimization of the humanized framework might be possible through additional mutations. Of the Vernier residues identified by Foote & Winter *J. Mol. Biol.* 224:487-499 (1992), only residues V_L 46, V_H 2 and V_H 48 differed in the A4.6.1 versus human $V_L\kappa$I-V_HIII framework (Figs. 5A and 5B) but were not randomized in our phagemid library. A molecular model of the humanized A4.6.1 Fv fragment showed that V_L 46 sits at the V_L-V_H interface and could influence the conformation of CDR-H3. Furthermore, this amino acid is almost always leucine in most $V_L\kappa$ frameworks (Kabat *et al.*, *supra*), but is valine in A4.6.1. Accordingly, a Leu -> Val substitution was made at this position in the background of hu2.10. Analysis of binding kinetics for this new variant, hu2.10V, indicated a further 6-fold improvement in the K_D for VEGF binding, demonstrating the importance of valine at position V_L 46 in antibody A4.6.1. The K_D for hu2.10V (9.3 nM) was thus within 6-fold that of the chimera. In contrast to V_L 46, no improvement in the binding affinity of hu2.10 was observed for replacement of either V_H 2 or V_H 48 with the corresponding residue from murine A4.6.1.

EXAMPLE 3

In this example, CDR randomization, affinity maturation by monovalent Fab phage display, and cumulative combination of mutations were used to enhance the affinity of a humanized anti-VEGF antibody.

Construction of Humanized Antibody pY0101: Phage-displayed antibody vector phMB4-19-1.6 (see Figs. 8A-E) was used as a parent. In this construct, anti-VEGF is expressed as a Fab fragment with its heavy chain fused to the N-terminus of the truncated g3p. Both the light and heavy chains are under the control of phoA promoter with an upstream stII signal-sequence for secretion into the periplasm. Point mutations outside the CDR regions were made by site-directed mutagenesis to improve affinity for VEGF with

oligonucleotides HL-242, HL-243, HL-245, HL-246, HL-254, HL-256, and HL-257 as shown in Table 8 below:

Table 8: Oligos for Directed Mutations

Oligo Number	Region	Substitution/ Comments	Sequence
HL-242	VL	M4L	5'-GATATCCAGTTGACCCAGTCCCCG-3' (SEQ ID NO:29)
HL-243	VL	L46V	5'-GCTCCGAAAGTACTGATTTAC-3' (SEQ ID NO:30)
HL-245	VH	CDR-7	5'-CGTCGTTTCACTTTTTCTGCAGACACCT CCAGCAACACAGTATACCTGCAGATG-3' (SEQ ID NO:31)
HL-246	VH	R98K	5'-CTATTACTGTGCAAAGTACCCCCAC-3' (SEQ ID NO:32)
HL-254	VL	Y71F	5'-GGGACGGATTTCACTCTGACCATC-3' (SEQ ID NO:33)
HL-256	VH	I37V	5'-GGTATGAACTGGGTCCGTCAGGCCCC-3' (SEQ ID NO:34)
HL-257	VH	CDR-7 A72L S76K N77S	5'-CGTCGTTTCACTTTTTCTTTAGACACCT CCAAAAGCACAGCATACCTGCAGATGAA C-3' (SEQ ID NO:35)

The resulting variant was termed Y0101 (Figs. 9A and 9B).

Construction of the First Generation of Antibody-Phage Libraries: To prevent contamination by wild-type sequence, templates with the TAA stop codon at the targeted sites for randomization were prepared and used for constructing libraries by site-directed mutagenesis with oligonucleotides using the degenerate NNS codon (where N is an equal mixture of A, G, C, and T while S is an equal mixture of G and C) for saturation mutagenesis. VL1 and VH3 were chosen as potential candidates for affinity enhancement (Figs. 9A and B). Within the CDRs, two libraries were constructed from the pY0101 template. VL1 was mutated using stop-template oligonucleotides HL-248 and HL-249 (Table 9) and library oligonucleotides HL-258 and HL-259 (Table 10). Similarly, three libraries were constructed for VH3 using stop template oligonucleotides HL-250, HL-251, and HL-252 (Table 9), and library oligonucleotides HL-260, HL-261, and HL-262 (Table 10). Library construction is summarized in Tables 9 and 10 below.

Table 9: Template Oligos for Mutagenesis

Oligo Number	Region Comments	Sequence
HL-248	VL1	5'-GGGTCACCATCACCTGCTAAGCATAATAATAA TAAAGCAACTATTTAAACTGG-3' (SEQ ID NO:36)
HL-249	VL1	5'-GCGCAAGTCAGGATATTTAATAATAATAATAA TGGTATCAACAGAAACCAGG-3' (SEQ ID NO:37)
HL-250	VH3	5'-GTCTATTACTGTGCAAAGTAATAACACTAATA AGGGAGCAGCCACTGG-3' (SEQ ID NO:38)
HL-251	VH3	5'-GGTACCCCCACTATTATTAATAATAATAATGG TATTTCGACGTCTGGGG-3' (SEQ ID NO:39)
HL-252	VH3	5'-CACTATTATGGGAGCAGCCACTAATAATAATA AGTCTGGGTCAAGGAACCCTG-3' (SEQ ID NO:40)
HL-263	VH1	5'-TCCTGTGCAGCTTCTGGCTAATAATTCTAATA ATAAGGTATGAACTGGGTCCG-3' (SEQ ID NO:41)
HL-264	VH2	5'-GAATGGGTTGGATGGATTAACTAATAATAAG GTTAACCGACCTATGCTGCGG-3' (SEQ ID NO:42)
YC-80	VH3	5'-CTGTGCAAAGTACCCGTAATATTAATAATAAT AACACTGGTATTTCGAC-3' (SEQ ID NO:43)
YC-100	CDR7	5'-CGTTTCACTTTTTCTTAAGACTAATCCAAATA AACAGCATACCTGCAG-3' (SEQ ID NO:44)
YC-102	VH2	5'-GAATGGGTTGGATGGATTTAATAATAATAAG GTGAACCGACCTATG-3' (SEQ ID NO:45)

Table 10: Random Oligos for Library Construction

Oligo Number	Region Comment	Sequence
HL-258	VL1	5'-GGGTCACCATCACCTGCNNSGCANNSNNSNNSNN SAGC AACTATTTAAACTGG-3' (SEQ ID NO:46)
HL-259	VL1	5'-GCGCAAGTCAGGATATTNNSNNSNNSNNSNNSTG GTATCAACAGAAACCAGG-3' (SEQ ID NO:47)
HL-260	VH3	5'-GTCTATTACTGTGCAAAGNNSNNSCACNNSNNSG GGAGCAGCCACTGG-3' (SEQ ID NO:48)
HL-261	VH3	5'-TACCCCCACTATTATNNSNNSNNSNNSTGGTATTT CGACGTCTGGGG-3' (SEQ ID NO:49)
HL-262	VH3	5'-CACTATTATGGGAGCAGCCACNNSNNSNNSNNSG TCTGGGGTCAAGGAACCCTG-3' (SEQ ID NO:50)
HL-265	VH1	5'-TCCTGTGCAGCTTCTGGCNNSNNSTTCNNSNNSN NSGGTATGAACTGGGTCCG-3' (SEQ ID NO:51)
HL-266	VH2	5'-GAATGGGTTGGATGGATTAACNNSNNSNNSGGTN NSCCGACCTATGCTGCGG-3' (SEQ ID NO:52)
YC-81	VH3	5'-CTGTGCAAAGTACCCGNNSTATNNSNNSNNSNNS CACTGGTATTTCGAC-3' (SEQ ID NO:53)
YC-101	CDR7	5'-CGTTTCACTTTTTCTNNSGACNNSTCCAAANNSA CAGCATACCTGCAG-3' (SEQ ID NO:54)
YC-103	VH2	5'-GAATGGGTTGGATGGATTNNSNNSNNSNNSGGTG AACCGACCTATG-3' (SEQ ID NO:55)

The products of random mutagenesis reactions were electroporated into XL1-Blue *E.coli* cells (Stratagene) and amplified by growing 15-16 h with M13KO7 helper phage. The complexity of each library, ranging from 2 x 10^7 to 1.5 x 10^8, was estimated based upon plating of the initial transformation onto carbenicillin plates.

Initial Affinity Selections: For each round of selection, approximately 10^9-10^{10} phage were screened for binding to plates (Nunc Maxisorp 96-well) coated with 2 µg/mL VEGF (recombinant; residue 9-109 version) in 50 mM carbonate buffer, pH 9.6 and blocked with 5% instant milk in 50 mM carbonate buffer, pH 9.6. After 1-2 hour binding at room temperature, in the presence of 0.5% bovine serum albumin and 0.05% TWEEN 20™ in PBS, the phage solution was removed, and the plate was washed ten times with PBS/TWEEN™ (0.05% TWEEN 20™ in PBS buffer). Typically, to select for enhanced

affinity variants with slower dissociation rates, the plates were incubated with PBS/TWEEN™ buffer for a period of time which lengthened progressively for each round of selection (from 0 minute for the first round, to 3 h for the ninth round of selection). After the PBS/TWEEN™ buffer was removed, the remained phages were eluted with 0.1 M HCl and immediately neutralized with 1/3 volume of 1 M Tris, pH 8.0. The eluted phages were propagated by infecting XL1-Blue *E.coli* cells (Stratagene) for the next selection cycle.

Sequencing data revealed that both VL1 libraries, even after the eighth/ninth round of sorting, remained diverse, tolerating various type of residues at the sites of randomization. In contrast, the VH3 libraries retained only wild type residues or had very conservative substitutions. This suggested that the VL1 was more exposed to solvent and lay outside the binding interface. In contrast, VH3 did not show dramatically different sidechain substitutions, and therefore might be more intimately involved in antigen binding.

Phage-ELISA Assay of Binding Affinities: From each of these libraries, representative clones (those represented by abundant sequences) were assayed for their affinities relative to that of parent clone pY0101 in a phage-ELISA assay. In such an assay, phages were first serially diluted to determine a fractional saturation titer which was then held constant and used to incubate with varying concentrations of VEGF (starting at 200 nM to 0 nM) in solution. The mixture was then transferred onto plate precoated with VEGF (2 μg/mL) and blocked with 5% instant milk, and allowed to equilibrate for 1 hour at room temperature. Thereafter, the phage solution was removed and the remaining bound phages were detected with a solution of rabbit anti-phage antibody mixed with goat anti-rabbit conjugate of horse radish peroxidase. After an hour incubation at room temperature, the plate was developed with a chromogenic substrate, *o*-phenylenediamine (Sigma). The reaction was stopped with addition of ½ volume of 2.5 M H_2SO_4. Optical density at 492nm was measured on a spectrophotometric plate reader.

Although all of the selected clones from these five libraries showed either weaker or similar affinities than that of wild type pY0101 in phage-ELISA assay, one particular variant (pY0192) from library HL-258 displayed an apparent advantage (about 10 fold) in the level of expression or phage display relative to pY0101. This clone contained mutations S24R, S26N, Q27E, D28Q, and I29L in the VL region (Fig. 9A). In addition, this variant was found to have a spurious mutation, M34I, in VH. This variant showed no significant difference in binding affinity to VEGF as compared with the pY0101 variant. To improve

the level of Fab-display on phage, and the signal-to-noise ratio for phage-ELISA assays, the corresponding substitutions in pY0192 at VL1 were incorporated into the template background for constructing both CDR Ala-mutants and the second generation of anti-VEGF libraries.

Ala-Scanning the CDRs of Anti-VEGF: To determine the energetics contributed by each of the amino acids in the CDR regions and thus better select target residues for randomization, the CDR regions were screened by substituting alanine for each residue. Each Ala mutant was constructed using site-directed mutagenesis with a synthetic oligonucleotide encoding for the specific alanine substitution. Where Ala was the wild-type residue, Ser was substituted to test the effect of a sidechain substitution. Phage clones having a single Ala mutation were purified and assayed in phage-ELISA as described above. Results of the Ala-scan demonstrated that Ala-substitution at various positions can have an effect, ranging from 2 to > 150 fold reductions, on antigen binding affinity compared to pY0192. In addition, it confirmed a previous observation that VH3, but not VL1, was involved in antigen binding. Results of the CDR Ala-scan are summarized in Table 11 below.

Table 11: Relative VEGF Affinities of Ala-Scan Fab Variants

Residue VL	IC50 (mut) / IC50 (wt)	Residue VH	IC50 (mut) / IC50 (wt)
R24A	1	G26A	2
A25S	1	Y27A	34
N26A	1	T28A	1
E27A	1	F29A	16
Q28A	1	T30A	1
L29A	1	N31A	>150
S30A	2	Y32A	>150
N31A	2	G33A	6
Y32A	2	I34A	6
L33A	2	N35A	66
N34A	4		
		W50A	>150
F50A	1	I51A	4
T51A	1	N52A	>150
S52A	1	T53A	9
S53A	1	Y54A	9
L54A	1	T55A	4
H55A	1	G56A	1

Residue VL	IC50 (mut) / IC50 (wt)	Residue VH	IC50 (mut) / IC50 (wt)
S56A	1	E57A	2
		P58A	1
Q89A	4	T59A	3
Q90A	3	Y60A	2
Y91A	14	A61S	1
S92A	1	A62S	1
T93A	1	D63A	1
V94A	2	F64A	1
P95A	3	K65A	1
W96A	>150	R66A	1
T97A	1		
		Y99A	>150
		P100A	38
		H101A	4
		Y102A	4
		Y103A	5
		G104A	2
		S105A	1
		S106A	>150
		H107A	2
		W108A	>150
		Y109A	19
		F110A	25
		D111A	2

All variants are in the background of pY0192 ("wt"; see Figs. 9A-B). IC50's were determined in a competitive phage-ELISA assay.

The largest effects of Ala substitutions are seen in CDRs H1, H2, and H3, including Y27A (34-fold reduction in affinity), N31A, Y32A, W50A, N52A, Y99A, S106A and W108A (each >150-fold reduction); N35A (66-fold reduction), P100A (38-fold reduction) and F110A (25-fold reduction). In contrast, only one VL substitution had a large impact on binding affinity, W96A (>150-fold reduction). These results point to the three VH CDRs as the main energetic determinants of Fab binding to VEGF, with some contribution from VL3.

Design of Second-Generation CDR Mutation Libraries: Two additional libraries which randomized existing residues in anti-VEGF version Y0192 were designed based upon inspection of the crystal structure. In VH2, residues 52-55 were randomized because they

lie within the binding interface with VEGF. An additional region of the Fab, termed "CDR7" (see Fig. 10B), was also targeted for randomization because several residues in this loop, while not contacting VEGF, do have contacts with the VH loops of the antibody. These represented potential sites for affinity improvement through secondary effects upon the interface residues. Residues L72, T74, and S77 were randomized in this CDR7 library.

Also based upon the crystal structure, one of the original CDR libraries was reconstructed to re-test the potential for affinity maturation in the VH1 CDR. Residues 27, 28, and 30-32 were randomized using the new Y0192 background.

Second-Generation Selections of Anti-VEGF Libraries: Based on Ala-scan results as well as the crystal structure of the antigen-antibody (F(ab)-12) complex, a total of seventeen libraries were constructed using the pY0192 template and stop-template oligonucleotides (which code for a stop codon at the sites targeted for randomization) YC-80, YC-100, YC-102, HL-263, and HL-264 (Table 9 above). The corresponding randomization oligonucleotides (which employ NNS at the sites targeted for randomization) were YC81, YC-101, YC-103, HL-265, and HL-266 (Table 10 above). The resulting transformants yielded libraries with complexities ranging from 6×10^7 to 5×10^8 which suggests that the libraries were comprehensive in covering all possible variants. Phage libraries were sorted for 7-8 rounds using conditions as described in Table 12 below.

Table 12: Conditions for Secondary Selections of Fab Variants

Round of Selection	Incubation Time (hr)	Incubation Solution	Incubation Temp. (°C)
1	0	0	room temp.
2	1	ELISA buffer	room temp.
3	2	1 μM VEGF/ELISA	room temp.
4	18	1 μM VEGF/ELISA	room temp.
5	37	1 μM VEGF/ELISA	room temp.
6	17 hr @ room temp./ 30 hr @ 37°C	1 μM VEGF/ELISA	room temp./37°C
7	63	1 μM VEGF/ELISA	37°C
8	121	1 μM VEGF/ELISA	37°C

ELISA buffer contained 0.5% bovine serum albumin and 0.05% TWEEN 20™ in PBS. VEGF was included in the incubation buffer to minimize rebinding of phages to VEGF coated on the surface of the plate. Sorting of these libraries yielded phage enrichments over 7 to 8 rounds of selection.

Phage-ELISA Assays of Second Generation Clones: After eight round of selections, ten to twenty clones from each library were isolated from carbenicillin containing plates harboring *E. coli* (XL1) colonies which had been infected with an eluted phage pool. Colonies were isolated and grown with helper phage to obtain single-stranded DNA for sequencing. CDR substitutions selected for more favorable binding to VEGF were deduced from the DNA sequences of phagemid clones. A sampling of selected clones is shown in Table 13 below.

Table 13: Protein Sequences of Anti-VEGF Variants from Second Generation Fab-Phage Libraries

Variants from library YC-81	
Name	**VH3 sequence (residues 99-111)**
Y0238-1	YPYYRGTSHWYFD (SEQ ID NO:56)
Y0238-2	YPYYINKSHWYFD (SEQ ID NO:57)
Y0238-3	YPYYYGTSHWYFD (SEQ ID NO:58)
Y0238-4	YPYYYNQSHWYFD (SEQ ID NO:59)
Y0238-5	YPYYIAKSHWYFD (SEQ ID NO:60)
Y0238-6	YPYYRDNSHWYFD (SEQ ID NO:61)
Y0238-7	YPYYWGTSHWYFD (SEQ ID NO:62)
Y0238-8	YPYYRQNSHWYFD (SEQ ID NO:63)
Y0238-9	YPYYRQSSHWYFD (SEQ ID NO:64)
Y0238-10	YPYYRNTSHWYFD (SEQ ID NO:65)
Y0238-11	YPYYKNTSHWYFD (SEQ ID NO:66)
Y0238-12	YPYYIERSHWYFD (SEQ ID NO:67)
Y0228-21	YPYYRNASHWYFD (SEQ ID NO:68)
Y0228-22	YPYYTTRSHWYFD (SEQ ID NO:69)
Y0228-23	YPYYEGSSHWYFD (SEQ ID NO:70)

Y0228-24	YPYYRQRGHWYFD (SEQ ID NO:71)
Y0228-26	YPYYTGRSHWYFD (SEQ ID NO:72)
Y0228-27	YPYYTNTSHWYFD (SEQ ID NO:73)
Y0228-28	YPYYRKGSHWYFD (SEQ ID NO:74)
Y0228-29	YPYYTGSSHWYFD (SEQ ID NO:75)
Y0228-30	YPYYRSGSHWYFD (SEQ ID NO:76)
Y0229-20	YPYYTNRSHWYFD (SEQ ID NO:77)
Y0229-21	YPYYRNSSHWYFD (SEQ ID NO:78)
Y0229-22	YPYYKESSHWYFD (SEQ ID NO:79)
Y0229-23	YPYYRDASHWYFD (SEQ ID NO:80)
Y0229-24	YPYYRQKGHWYFD (SEQ ID NO:81)
Y0229-25	YPYYKGGSHWYFD (SEQ ID NO:82)
Y0229-26	YPYYYGASHWYFD (SEQ ID NO:83)
Y0229-27	YPYYRGESHWYFD (SEQ ID NO:84)
Y0229-28	YPYYRSTSHWYFD (SEQ ID NO:85)
Variants from library HL-265	
Name	**VH1 sequence (residue 26-35)**
Y0243-1	GYDFTHYGMN (5/10 clones) (SEQ ID NO:86)
Y0243-2	GYEFQHYGMN (SEQ ID NO:87)
Y0243-3	GYEFTHYGMN (SEQ ID NO:88)
Y0243-4	GYDFGHYGMN (SEQ ID NO:89)
Y0243-5	GYDFSHYGMN (SEQ ID NO:90)
Y0243-6	GYEFSHYGMN (SEQ ID NO:91)
Variants from library YC-101	
Name	**VH "CDR7" sequence (residues 70-79)**
Y0244-1	FSVDVSKSTA (SEQ ID NO:92)
Y0244-2	FSLDKSKSTA (SEQ ID NO:93)
Y0244-3	FSLDVWKSTA (SEQ ID NO:94)
Y0244-4	FSIDKSKSTA (:95)

The sequence of the randomized region only is shown as deduced from DNA sequencing.

When a number of clones were tested along with the parent clone pY0192 in phage-ELISA assay, none showed a distinctive improvement over the parental clone. This could be explained by the time-scale on which the assay was performed (< 3 hours).

In order to quantify improvement in antigen binding over parent clone, several anti-VEGF variants' DNA were transformed into *E. coli* strain 34B8, expressed as Fab, and purified by passing the periplasmic shockate through a protein G column (Pharmacia) as described in Example 2 above.

CDR Combination Variants: To improve VEGF binding affinity further, mutations found by phage display were combined in different CDRs to create multiple-CDR mutants. In particular, the mutations identified in the most affinity-improved phage variants from VH1, VH2, and VH3 libraries were combined (Table 14) in order to test for additivity of their contributions to binding affinity.

Table 14: Combination CDR Anti-VEGF Variants

Name	Parent clone	Mutagenesis oligo/ comments	Sequence
Y0313-1	Y0243-1	YC-115 (VH3: H101Y and S105T)	5'-GCAAAGTACCCGTACTATTATGGGACGAGCCACTGGTATTTC-3' (SEQ ID NO:96)
Y0317	Y0313-1	YC-108 (revert VL1 back to wild type)	5'-GTCACCATCACCTGCAGCGCAAGTCAGGATATTAGCAACTATTTAAAC-3' (SEQ ID NO:97)
Y0313-3	Y0238-3	YC-116 (VH3; T105S)	5'-CCGTACTATTATGGGAGCAGCCACTGGTATTTC-3' (SEQ ID NO:98)

Mutations from the indicated parental vectors were combined with those from the indicated oligonucleotide by site-directed mutagenesis to yield the combination variants listed.

Version Y0317 is equivalent to Y0313-1 except that the background mutation in VL1 was removed and its sequence reverted back to that in pY0101. The effects of

mutating H101Y and S105T were tested by constructing a reversion mutant from Y0238-3.

BIAcore Analysis: The VEGF-binding affinities of Fab fragments were calculated from association and dissociation rate constants measured using a BIAcore-2000™ surface plasmon resonance system (BIAcore, Inc., Piscataway, NJ). A biosensor chip was activated for covalent coupling of VEGF using N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) according to the supplier's (BIAcore, Inc., Piscataway, NJ) instructions. VEGF was buffered exchanged into 20 mM sodium acetate, pH 4.8 and diluted to approximately 50 µg/mL. An aliquot (35 µL) was injected at a flow rate of 2 µL/min to achieve approximately 700-1400 response units (RU) of coupled protein. Finally, 1 M ethanolamine was injected as a blocking agent.

For kinetics measurements, two-fold serial dilutions of Fab were injected in PBS/TWEEN™ buffer (0.05% TWEEN 20™ in phosphate buffered saline) at 25°C at a flow rate of 10 µL/min. On rates and off rates were calculated using standard protocols (Karlsson *et al. J. Immun. Methods* 145:229-240 (1991)). Equilibrium dissociation constants, Kd's from surface plasmon resonance (SPR) measurements were calculated as koff/kon. Data are shown in Table 15 below.

Table 15: Kinetics of Fab-VEGF binding from BIAcore™ measurements

Variant	Kon (10^4/M/s)	koff (10^{-4}/s)	Kd (nM)	Kd (wt) / Kd (mut)
Y0244-1	3.4	2.7	8	3.6
Y0244-4	5.2	1.7	3.3	0.9
Y0243-1	6.7	0.45	0.7	4.1
Y0238-3	1.7	≤0.04*	≤0.2*	≥14*
Y0238-7	1.5	≤0.06*	≤0.4*	≥7.3*
Y0238-10	1.6	0.09	0.6	4.8
Y0238-5	0.8	0.08	0.9	3.2
Y0238-1	2.6	0.09	0.4	7.3
Y0313-1	3.5	≤0.054*	≤0.15*	≥20*

Table 15: Kinetics of Fab-VEGF binding from BIAcore™ measurements				
Y0313-3	1.2	0.081	0.65	4.5

* The dissociation rate observed probably reflects an upper limit for the true dissociation rate in these experiments, since the off-rate is approaching the limit of detection by BIAcore.

The BIAcore™ data in Table 15 show that several variants had improved affinity over Y0192. For example, a CDRH1 variant, Y0243-1, showed 4.1 fold enhanced affinity, arising from mutations T28D and N31H. Variant Y0238-3 showed at least a 14 fold improvement in binding affinity over Y0192. Both CDRH3 mutations contribute to the improved affinity of Y0238-3 because reversion of T105 to S (variant Y0313-3) reduces the affinity of Y0238-3 from 0.15nM to 0.65 nM (see Table 15). The greater affinity enhancement relative to Y0192 was seen for Y0313-1, which contained CDRH3 mutations combined with CDRH1 mutations.

Cell-Based Assay of VEGF Inhibition: Several versions of the A4.6.1 anti-VEGF antibody were tested for their ability to antagonize VEGF (recombinant; version 1-165) in induction of the growth of HuVECs (human umbilical vein endothelial cells). The 96-well plates were seeded with 1000 HuVECs per well and fasted in assay medium (F12:DMEM 50:50 supplemented with 1.5% diafiltered fetal bovine serum) for 24 h. The concentration of VEGF used for inducing the cells was determined by first titrating for the amount of VEGF that can stimulate 80% of maximal DNA synthesis. Fresh assay medium containing fixed amounts of VEGF (0.2 nM final concentration), and increasing concentrations of anti-VEGF Fab or Mab were then added. After 40 h of incubation, DNA synthesis was measured by incorporation of tritiated thymidine. Cells were pulsed with 0.5 μCi per well of [3H]-thymidine for 24 h and harvested for counting, using a TopCount gamma counter.

The results (Fig. 11) show that the full-length IgG form of F(ab)-12 was significantly more potent in inhibiting VEGF activity than the Fab form (here, Y0192 was used). However, both variants Y0238-3 and Y0313-1 showed even more potent inhibition of VEGF activity than either the Y0192 Fab or F(ab)-12 Mab. Comparing the Fab forms, variant Y0313-1 appeared >30-fold more potent than the wild-type Fab. It should be noted that the amount of VEGF (0.2 nM) used in this assay is potentially limiting for determination of an accurate IC50 for the mutant. For example, if the binding affinity (Kd) of the mutant is in fact < 0.2 nM, the IC50 in this experiment will appear higher than under conditions of